ATF3 expression improves motor function in the ALS mouse model by promoting motor neuron survival and retaining muscle innervation

Rhona Seijffers,1,2,3 Jiangwen Zhang,4 Jonathan C. Matthews,5 Adam Chen,6,7 Eric Tamrazian,8,9,10 Olusegun Babaniyi,11,12,13,14 Martin Selig,15,16 Meri Hynynen,17 Clifford J. Woolf,18,19,20 and Robert H. Brown, Jr.21

1To whom correspondence should be addressed. E-mail: rseijffers@gmail.com.
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ALS is a fatal neurodegenerative disease characterized by a progressive loss of motor neurons and atrophy of distal axon terminals in muscle, resulting in loss of motor function. Motor end plates denervated by axonal retraction of dying motor neurons are partially reinnervated by remaining viable motor neurons; however, this axonal sprouting is insufficient to compensate for motor neuron loss. Activating transcription factor 3 (ATF3) promotes neuronal survival and axonal growth. Here, we reveal that forced expression of ATF3 in motor neurons of transgenic SOD1G93A ALS mice delays neuromuscular junction denervation by inducing axonal sprouting and enhancing motor neuron viability. Maintenance of neuromuscular junction innervation during the course of the disease in ATF3/SOD1G93A mice is associated with a substantial delay in muscle atrophy and improved motor performance. Although disease onset and mortality are delayed, disease duration is not affected. This study shows that adaptive axonal growth-promoting mechanisms can substantially improve motor function in ALS and importantly, that augmenting viability of the motor neuron soma and maintaining functional neuromuscular junction connections are both essential elements in therapy for motor neuron disease in the SOD1G93A mice. Accordingly, effective protection of optimal motor neuron function requires restoration of multiple dysregulated cellular pathways.

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Significance

This study reports on the beneficial effects of forcing high expression of the transcription factor activating transcription factor 3 (ATF3) in ALS. ALS is a noncurable adult-onset disease that attacks motor neurons, resulting in paralysis and death. ATF3 overexpression in motor neurons in an ALS mouse model modifies gene expression and drives the neurons into a prosurvival and proregenerative state, increasing motor neuron survival and maintaining axonal integrity and NMJ innervation. ATF3 expression in SOD1G93A mice resulted in a delay in disease deterioration that manifested in delayed muscle atrophy, improved muscle performance, and a small increase in lifespan.

Author contributions: R.S., C.J.W., and R.H.B. designed research; R.S., J.C.M., A.C., E.T., O.B., M.S., and M.H. performed research; R.S. and J.Z. analyzed data; and R.S., C.J.W., and R.H.B. wrote the paper.

The authors declare no conflict of interest.

1To whom correspondence should be addressed. E-mail: rseijffers@gmail.com.
2C.J.W. and R.H.B. contributed equally to this work.

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Results

ATF3 Promotes Motor Neuron Survival in ATF3/SOD1G93A Mice. To address the effect of forced ATF3 expression on motor neuron survival in the SOD1 mutant mice, ATF3 transgenic mice (19) were crossed with hSOD1G93A transgenic mice that harbor the mutant gene at high copies (6), and their progeny was analyzed. These ATF3 transgenic mice express the ATF3 transgene under control of the thy1.2 promoter (19), which drives transgene expression specifically in postnatal neurons (37). To exclude transgene expression variability, two independent ATF3 transgenic founder lines that express ATF3 abundantly in most spinal motor neurons were crossed with SOD1G93A mice and generated identical results.

Progressive motor neuron cell death is a hallmark of ALS. Motor neuron survival was quantified at 60 d of age (a presymptomatic stage), 90 d of age (when disease onset is apparent), and 120 d of age (by which time the mice show clear muscle weakness but are not at end stage [130–140 d of age]). Large lumbar motor neurons with a cell body area of ≥450 μm² were quantified using the neuronal marker NeuN. Thus, only the large α-motor neurons most vulnerable to cell death in ALS (2, 38, 39) were quantified, and the γ-type motor neurons that are resistant to the disease were excluded. In SOD1G93A jittermates at the presymptomatic stage, no motor neuron cell death was detected; but by the time of disease onset, only 45.8% of motor neurons had survived, with 19.3% of motor neurons surviving at 120 d of age (Fig. 1A and D–F). In contrast, motor neuron survival in the ATF3/SOD1G93A mice was substantially higher; 78.6% and 57.6% of the motor neurons survived at 90 and 120 d, respectively (Fig. 1A and H–J).

Less than 10% of large motor neurons in SOD1G93A mice induced endogenous ATF3 expression, which was detected by double immunostaining for ATF3 and NeuN (Fig. 1B–F). As anticipated, in the presence of the ATF3 transgene, 79.71 ± 2.14% and 77.53 ± 2.8% of the motor neurons were ATF3-positive in ATF3/SOD1G93A and ATF3/WT mice, respectively (Fig. 1B and G–J). The fraction of non-ATF3–expressing motor neurons (~20%) in ATF3/WT transgenic mice and in ATF3/SOD1G93A mice was similar. This fraction in ATF3/SOD1G93A mice remained unaltered over time (Fig. 1B and H–J). A cell-autonomous mechanism of ATF3 action would be expected to enhance the survival of only ATF3-positive neurons and thus, lead to a decrease in the ATF3-negative fraction over time. However, the lack of change in this fraction suggests that the beneficial influence of ATF3 may also be mediated by non-cell-autonomous actions that protect neighboring motor neurons that do not express ATF3 (Fig. 1B and H–J). For example, a non-cell-autonomous action could be achieved if ATF3 induces the expression of secreted factors that then exert the protective action on the non-ATF3–expressing cells or because of reduced neuroinflammation caused by the increased neuronal survival.

ATF3 Delays the Loss of Axonal Integrity in ATF3/SOD1G93A Mice. Analysis of lumbar L5 ventral roots (Fig. 2) revealed axonal damage early on at the presymptomatic stage (60 d) in SOD1G93A mice. Large-caliber α-axons with a diameter >3.5 μm degenerate in ALS. This degeneration is observed in the ventral root as a decline in diameter size. A continuous decline in large axons with a corresponding increase in the number of small axons was observed over time in SOD1G93A mice (Fig. 2). In contrast, significant axonal damage in the ventral roots was detected only at 120 d in ATF3/SOD1G93A mice (Fig. 2F). The most extreme differences were detected at 90 d, at which time ATF3/SOD1G93A axons remained intact; by contrast, in SOD1G93A mice at this time point, there was an increase of 51% in small-caliber axons because of the rapid degeneration of SOD1G93A large-caliber axons (Fig. 2C–F).

ATF3 Delays NMJ Denervation and Muscle Atrophy. Evaluation of NMJs in the gastrocnemius muscle of SOD1G93A mice revealed denervation as early as 60 d of age, with only 70.5 ± 4.6% of the NMJs remaining innervated at this time (Fig. 3 C and K). Denervation of the NMJs accelerated in SOD1G93A mice as the disease progressed, and it was accompanied by a collapse of the NMJ structures, which were revealed by a reduction in the number of pretzel-shaped NMJs (Fig. 3L). NMJ collapse was apparent at 90 d in SOD1G93A mice and rapidly accelerated, with only 56.6% of NMJs retaining their normal morphology in the gastrocnemius muscle at 120 d (Fig. 3 L and I).

The rate of NMJ denervation extrapolated from Fig. 3K does not include the acceleration in NMJ collapse. Evaluation of the actual percent of NMJ denervation, which takes into account the loss of NMJs, reached 69.7 ± 5.2% at 120 d in SOD1G93A mice (Fig. 3M). In contrast, denervated NMJs were detected only at 120 d of age in ATF3/SOD1G93A mice; even then, 83.1 ± 3.4% of the NMJs remained innervated, and there was no apparent NMJ collapse (Fig. 3 H, J, K, and M). No difference in the extent of NMJ innervation between WT and ATF3/WT littermates was detected, indicating that ATF3 in WT mice did not induce hyperinnervation under normal conditions (Fig. 3 A, B, and K).

Measurements of gastrocnemius muscle weight in the SOD1G93A mice revealed muscle wasting (24% weight loss) at the presymptomatic stage (60 d), correlating with the early NMJ denervation detected at this time (Figs. 3 and 4). The decline in muscle mass in SOD1G93A mice advanced over time; weight...
In ATF3/SOD1G93A mice, terminal sprouting was also first detected at 90 and 120 d of age, with no increase over time (Fig. 5D). The improved motor performance in ATF3/SOD1G93A mice directly reflected an impact of the ATF3 transgene on the disease; the ATF3 transgene did not enhance muscle strength in ATF3/WT transgenic littermates compared with WTs.

The improved motor performance in ATF3/SOD1G93A mice was prolonged by 7.7 d. The average ages at death were 137.2 ± 1.4 and 144.9 ± 2.1 d in the SOD1G93A and ATF3/SOD1G93A mice, respectively (P < 0.01 by Kaplan–Meier log rank test for survival) (Fig. 6C). However, no difference was identified in disease progression. ATF3/SOD1G93A double transgenic mice displayed increased muscle strength compared with SOD1G93A littermates as determined by the hind limb grip strength assay (Fig. 6A). The improved motor performance in ATF3/SOD1G93A mice directly reflected an impact of the ATF3 transgene on the disease; the ATF3 transgene did not enhance muscle strength in ATF3/WT transgenic littermates compared with WTs.

As the disease progressed, measurement of end plate innervation in SOD1G93A mice showed that motor axons retracted from the NMJs concomitantly with the development of muscle atrophy, which preceded motor neuron cell body death (Fig. 5A and B). Such axonal dying back phenomenon is well-characterized in both human ALS and mouse disease models (12). However, although some motor neuron cell death was already apparent in ATF3/SOD1G93A mice at 90 d of age, NMJs denervation was not detected in these mice until 120 d (Fig. 5A and C). This discrepancy suggests that, as NMJs are denervated by motor neuron loss, they become reinnervated by sprouts extending from the intact axons of surviving motor neurons, contributing to the delay in muscle atrophy (Fig. 5B). Extension of collateral and terminal axonal sprouts is detected in ALS disease, but this sprouting process is usually unable to compensate for loss of axonal connections with muscle (11). In SOD1G93A mice, quantification of terminal sprouts in the gastrocnemius muscle detected no sprouting at 60 d. The amount of terminal sprouting at 90 and 120 d was similar, with no increase over time (Fig. 5D). In ATF3/SOD1G93A mice, terminal sprouting was also first detected at 90 d and similar to the sprouting in SOD1G93A mice. However, at 120 d, the degree of terminal sprouting was significantly higher in ATF3/SOD1G93A mice than SOD1G93A mice (Fig. 5D–G). These data suggest that ATF3 promotes the capacity to form and maintain terminal axonal sprouts in SOD1 mutant mice to maintain NMJ innervation and prevent muscle atrophy.

**ATF3 Delays Motor Deficits and Disease Onset in ATF3/SOD1G93A Mice.**

We next tested whether the beneficial effects of ATF3 on motor neuron survival and axonal connectivity with muscle identified in this study had an impact on muscle performance and disease progression. ATF3/SOD1G93A double transgenic mice displayed increased muscle strength compared with SOD1G93A littermates as determined by the hind limb grip strength assay (Fig. 6A). The improved motor performance in ATF3/SOD1G93A mice directly reflected an impact of the ATF3 transgene on the disease; the ATF3 transgene did not enhance muscle strength in ATF3/WT transgenic littermates compared with WTs.

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duration between the groups ($P > 0.05$). Although ATF3 expression was sufficient to modestly delay the onset of the disease and consequently, prolong survival, disease duration was not extended. Nonetheless, as the disease progressed and SOD1G93A mice reached end stage, they were weak and largely immobile, whereas ATF3/SOD1G93A mice were in much better health. They were grooming and exploring their cages (Movies S1 and S2), illustrating the marked beneficial effects of ATF3 on muscle performance as the disease progressed.

**Fig. 4.** ATF3 expression in SOD1G93A mice delays muscle atrophy. (A) Representative image of the gastrocnemius muscle showing delayed muscle atrophy in ATF3/SOD1G93A compared with SOD1G93A mice at 120 d of age. (B and C) Muscle wasting was calculated by measurement of the gastrocnemius muscle weight in male mice littermates (the same trend was found in female mice). Data are presented in grams as mean ± SEM ($n = 4–6$ per group). *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$ by ANOVA with Bonferroni postanalysis. (C) Percent muscle atrophy was calculated relative to WT mice.

**Fig. 5.** Changes in architecture of the motor unit induced by SOD1G93A are attenuated by ATF3 transgene coexpression. (A) Comparison of progressive changes in percentages of remaining motor neurons (black diamonds), large axons (blue squares), innervated NMJs (extrapolated from the actual denervation data in Fig. 3M) (green triangles), and weight (red circles) in (Left) SOD1G93A and (Right) ATF3/SOD1G93A mice. In SOD1G93A mice, all parameters rapidly decline over time. By contrast, in ATF3/SOD1G93A mice, all four parameters are indistinguishable from WT at 60 d, and only numbers of large motor neurons declined by 90 d. At 120 d, all parameters are improved in ATF3/SOD1G93A mice compared with SOD1G93A counterparts. Arrow highlights the 90-d time point at which ATF3/SOD1G93A mice clearly show a decline in numbers of surviving motor neurons without a decline of innervated NMJs or other parameters. (B and C) A schematic model of the disease course illustrates the dying back phenomenon in SOD1G93A mice and induction of collateral sprouts in ATF3/SOD1G93A mice as a mechanism to maintain maximal NMJ innervation. Intact motor neurons and axons are in purple/blue. Diseased motor neurons are indicated with white specks, and degenerating axons are indicated with white and black. New collateral axonal sprouts are yellow; intact muscle is red, and NMJs are green. (D) Terminal sprouts were determined when the nerve extended beyond the acetylcholine receptor (AChR) clusters in the innervated NMJ at any direction. Sprouting is presented as the percent of NMJs with sprouts relative to the total number of innervated NMJs in each group. Increased sprouting is detected at 120 d in ATF3/G93A compared with G93A mice. *$P < 0.01$ by ANOVA with Bonferroni postanalysis ($n = 4–5$ mice per time point). (E–G) Immunostaining with $\alpha$-bungarotoxin (green) to label NMJs in the gastrocnemius muscle and antineurofilament (red) to mark innervating axons reveals sprouting at the NMJ in ATF3/G93A mice. The terminal sprout is indicated with an arrow.
Kaplan nerveation, despite some motor neuron loss, suggesting that degeneration, the ATF3/SOD1G93A mice showed little or no loss of muscle motor neurons that are in a proregenerative state. Moreover, at this by terminal and collateral sprouts extending from neighboring intact mice. (A) Hind limb grip strength analysis for WT, SOD1/WT, SOD1G93A, and ATF3/SOD1G93A mice. Data are mean ± SEM. *P < 0.05; ***P < 0.001 by repeated measures ANOVA with Bonferroni postanalysis (n = 30 mice per group). (B) and (C) ATF3/SOD1G93A mice display delayed onset of disease and prolonged survival (P = 0.0026 and P = 0.0013, respectively, by Kaplan–Meier log rank test for survival; n = 29 mice per group).

Discussion

This study shows that forced expression of the transcription factor ATF3 in SOD1G93A ALS mice slows the loss of motor neurons, delays terminal axonal atrophy, and augments axonal sprouting by driving the motor neurons into a prosurvival and regenerative state. In the ALS mouse model, forced ATF3 expression modifies the cell transcriptome (Figs. S3, S4, and S5) to support compensatory axonal sprouting and neuronal survival. At 90 d, the ATF3/SOD1G93A mice showed no loss of NMJ innervation, despite some motor neuron loss, suggesting that denervated NMJs in ATF3/SOD1G93A mice are rapidly reinnervated by terminal and collateral sprouts extending from neighboring intact motor neurons that are in a preregenerative state. Moreover, at this age, the ATF3/SOD1G93A mice showed little or no loss of muscle mass and showed improved muscle strength relative to SOD1G93A mice, such that the overall health of the animals was remarkably improved. However, although disease onset and death were slightly delayed, disease duration was not extended, indicating that induction of an intrinsic growth state in SOD1 mutant motor neurons by ATF3 was not sufficient to halt disease progression. Although ATF3-dependent compensation improved motor neuron viability, axonal dynamics, and motor performance, it did not arrest the ongoing motor neuron loss sufficiently to prevent death.

It is well-established that terminal axonal degeneration with NMJ denervation is an early component of motor neuron disease in ALS (12, 40, 41) (Fig. 5). Whereas intact motor neurons undergo distal axonal sprouting, this remodeling is insufficient to innervate neighboring denervated end plates and compensate for motor neuron loss, leading to progressive paralysis and death (11). Regardless of whether these deficits in axonal innervation or capacity to sprout arise as a consequence of events in the soma, such as excitotoxicity, endoplasmic reticulum stress, or mitochondrial pathology, or distally at peripheral nerve endings, it is evident from this study that the functional reserve provided by surviving motor neurons is enhanced by mechanisms that increase their axonal integrity, growth, and sprouting and thereby, maintain maximal connectivity with muscle.

The functional importance of the response of the axon to motor neuron pathology is illustrated by experimental paradigms that prevent death of the motor neuron soma. Three different experimental manipulations can delay motor neuron soma death in SOD1 mutant ALS mice: overexpression of the antiapoptotic protein Bel-2, KO of the proapoptotic Bax protein, and double KO of BAX and BAC (42–44). However, even in the BAX/BAC double KO mice (42), in which a substantial delay in motor neurons cell death is observed, axonal integrity is not preserved to the same degree, and the motor axon terminals continue to gradually die back from the NMJ. Despite the substantial preservation of motor neuron soma achieved in these mouse model experiments, motor neuron survival alone does not prevent axons from degenerating.

The strong propensity for axonal degeneration in SOD1 mutant ALS mice is shown in an additional model. In Wld* mice, a unique fusion protein of ubiquitin conjugation factor E4B and nicotinamide mononucleotide adenyltransferase 1 dramatically delays Wallerian degeneration after nerve injury and protects against axonal degeneration in several neuropathies and neurodegenerative models. However, when it is bred into the SOD1G93A mice, the Wld* gene fails to block axonal degeneration (45, 46). The molecular events controlled by Wld* expression that blunt axonal degeneration do not suffice to slow axonal degeneration triggered by mutant SOD1 protein. From this perspective, it is striking that, by contrast, the transcription factor ATF3 substantially augments axonal protection and sprouting, at least through ~90–100 d.

Mechanisms that act to protect only one compartment, either the motor neuron soma or the axons, do not seem sufficient to preserve a functional motor unit in ALS (42–46). The importance of both maintaining axonal connectivity with the muscle and protecting the motor neurons from cell death for preservation of a functional motor unit is clearly illustrated in this study. The modest degree of muscle denervation and atrophy at 120 d in ATF3/SOD1G93A mice is comparable with the denervation detected in SOD1G93A mice 2 mo earlier (at 60 d of age). However, muscle performance is not maintained to the same extent. This difference implies that newly collaterally innervated NMJs, although sufficient to prevent muscle atrophy, do not maintain full muscle strength as the disease progresses, which may reflect the limited function of collateral sprouts relative to normal inputs. The preservation of large α-motor axon integrity found in this study is crucial for maintaining muscle mass and function, but the fact that axonal damage and progression of motor neuron cell death are observed in ATF3/SOD1G93A mice at 120 d suggests that deleterious processes continue to impair motor neuron function over time and secondarily, may compromise the axonal maintenance, dynamic axonal sprouting, and remodeling that are promoted by ATF3. Future studies, including longitudinal observations on motor neuron survival, axonal remodeling, and NMJ function in ATF3/SOD1G93A and ATF3/ SOD1G93A mice with knockdown of the BAX/BAC proapoptotic gene may help tease out why the beneficial influence of neuroinflammation of motor neuron disease onset and lifespan is so moderate relative to the substantial effect that it has on reducing neuronal loss, axonal damage, and denervation of the NMJ. These studies may also help identify key disease-inducing pathways that are resistant to ATF3-mediated transcriptional changes. However, the early benefits of ATF3 on overall function are substantial and if they could be translated into patients, might dramatically improve quality of life. Additional interventions would be required to slow significantly the inexorable ATF3-resistant cell death.

Transcription factors control the expression of many genes, and therefore, they serve as good candidates to govern the synchronization and coordination between the many different gene pathways required to achieve regeneration and neuronal survival. ATF3, a basic leucine zipper transcription factor (14), regulates gene expression either directly by binding the DNA as a homodimer or heterodimer or indirectly by sequestering activators and repressors, and thus, its transcriptional targets depend on the specific cellular milieu. Neuronal expression of ATF3 induced favorable transcriptional changes in the ventral horn of ATF3/SOD1G93A mice (Figs. S3, S4, and S5), including reduced induction of neuroinflammatory and apoptotic gene pathways, improved nerve transmission, and enhanced expression of gene pathways involved in cytoskeleton organization, axon guidance, and neurogenesis. These findings suggest that ATF3 modifies the intrinsic growth state of the motor neurons to enable axonal
sprouting and remodeling in the ATF3/SOD1G93A mice (Figs. S4 and S5). Interestingly, the set of genes that is uniquely up-regulated in ATF3/SOD1G93A clusters in pathways that support axonal myelination and nerve transmission (Fig. S5), supporting a role for ATF3 in maintaining axonal integrity and function and thereby, retaining muscle mass and function.

This study (i) supports the view that neuroprotection to improve motor neuron viability will be required to slow disease progression and (ii) shows, in addition that, to counteract motor neuron loss, it may also be critical to induce regenerative mechanisms that reduce terminal axonal degeneration and enhance compensatory axonal sprouting and functional reinnervation of denervated NMJs. Because the cell survival and regenerative mechanisms that ATF3 induces are not sufficient to completely repair motor neurons in ALS, complementary protective mechanisms that target the multiple dysregulated cellular pathways will be required for full restoration, further reflecting the complexity of the disease.

### Materials and Methods

ATF3 transgenic mouse lines (19) that constitutively express ATF3 in neurons, including spinal motor neurons, were crossed with high-copy number SOD1G93A transgenic mice (6) (stock number 002726; Jackson Laboratories). Protocol for the behavioral examinations are described in SI Materials and Methods. Muscle, motor neuron, ventral root, and NMI analyses, including staining protocols and antibodies used, and additional methods are provided in SI Materials and Methods.

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18. Sejffers J, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG axonal myelination and nerve transmission (Fig. S5), supporting a role for ATF3 in maintaining axonal integrity and function and thereby, retaining muscle mass and function.
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Supporting Information

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SI Materials and Methods

Mouse Strains. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Children’s Hospital of Boston, University of Massachusetts Medical School, and Massachusetts General Hospital. This study used two activating transcription factor 3 (ATF3) transgenic mouse lines (19) (derived from two different founders) that constitutively express ATF3 in neurons, including spinal motor neurons, and were backcrossed at least seven generations into C57BL/6 mice. These mice were crossed with the high-copy number SOD1(G93A) transgenic mice (6) (stock number 002726; Jackson Laboratories). Mice progeny were distinguished by genotyping with specific primers for the human SOD1(G93A) mutant gene and primers specific for the ATF3 transgene. Progeny of four genotypes (WT, ATF3/WT, SOD1(G93A), and ATF3/SOD1(G93A)) of matched litters and gender was used unless otherwise indicated.

Evaluation of Disease Progression and Survival Rate. Disease onset, progression, and survival were monitored in the double transgenic ATF3/SOD1(G93A) compared with SOD1(G93A) mutant mice. All tests were performed blinded to the mouse genotype. The onset of the disease in the SOD1(G93A) mutant mice is at around 90 d of age; the disease progresses, and the mice die at ∼130–140 d. Examination of the mice began at ∼60 d of age. Mice were weighed and examined biweekly by lifting them up by their tails and viewing their hind limb flexibility, muscle tone, and weakness. At disease onset, the initial manifestation of weakness is a resting tremor of one limb (typically a hind limb). After onset was observed, mice were monitored daily for gait impairment and asymmetrical or symmetrical paralysis of the hind limbs. As the disease progresses, it eventually leads to death. Mice were euthanized when they were unable to right themselves within 15 s after being placed on their backs. All tests were performed blinded to the mouse genotype.

Grip Strength Analysis. Motor function/muscle strength was assayed biweekly beginning at 60 d of age using the grip strength test until the mice could no longer perform the task. The mice were trained to grip the bar of the grip strength apparatus (Chatillon) with their hind limbs, and then, they were pulled off the bar horizontally. Each measurement was performed in triplicate, and the force used to grip the bar was recorded in kilograms. All tests were performed blinded to the mouse genotype.

Motor Neuron Quantification. Mice were transcardially perfused with 4% (wt/vol) paraformaldehyde in phosphate buffer. The spinal cord lumbar region was excised and postfixed in 4% (wt/vol) paraformaldehyde in phosphate buffer, and then, it was cryoprotected in 30% (wt/vol) sucrose in PBS; 10-μm-thick sections were double stained for the neuronal marker Neun using a mouse monoclonal antibody at 1:2,000 dilution (Chemicon) and a rabbit polyclonal anti-ATF3 antibody at 1:200 (Santa Cruz) followed by a rabbit polyclonal antibody (Jackson ImmunoResearch). The ventral horn regions of eight nonadjacent sections (at least 600 μm apart per mouse) were used for the analysis. Motor neurons with clear cell body margins as detected by NeuN immunostaining with an area ≥ 450 μm² were quantified using Image J software. Only gender-matched littersmates were used at 60, 90, and 120 d of age. For each group, three to four mouse litters were used, with a total of five mice per time point quantified.

Neuromuscular Junction Analysis. The gastrocnemius muscle was excised, postfixed in 4% (wt/vol) paraformaldehyde in PBS overnight, and cryosectioned in 30% (wt/vol) sucrose in PBS; 20-μm-thick sections were double stained with α-bungarotoxin 488 conjugate at 1:1,000 dilution (Molecular Probes) and rabbit anti-neurofilament 200 at 1:1,000 dilution (Sigma) followed by a goat anti-rabbit antibody at 1:2,000 dilution (Jackson ImmunoResearch). All neuromuscular junctions with a pretzel-shaped morphology that stained positive for α-bungarotoxin binding to the acetylcholine receptor (AChR) were quantified on three nonadjacent sections at least 100 μm apart per mouse. Neuromuscular junctions were scored as innervated when both stains overlapped and scored as denervated when no neurofilament staining was detected. Gender-matched littersmates were used; four to five litters per gender with a total of nine mice per group and time point were quantified at 60, 90, and 120 d of age. Terminal sprouts were determined when the nerve extended beyond the AChR clusters in any direction.

Ventral Root Counts. Mice were perfused transcardially with 4% (wt/vol) paraformaldehyde in phosphate buffer, and the L5 ventral roots were excised and postfixed in 2.5% (vol/vol) glutaraldehyde. The tissue was then treated with 1% osmium tetroxide for 1 h, dehydrated through graded alcohols, and embedded in Epon plastic (EM Sciences); 1-μm-thick cross-sections were cut on an ultramicrotome, stained with toluidine blue, rinsed, and coverslipped. Images were captured, and the axons’ diameters were measured using the ImageJ software (National Institutes of Health). Three to four litters per gender with a total of six to nine mice per group per time point were quantified.

Statistical Analysis. One-way ANOVA was used to compare differences between groups over time. Kaplan–Meier log rank test for survival was used for comparing disease onset and lifespan between groups.

Microarray Analysis. Fresh frozen spinal cord lumbar region ventral horn samples of WT, ATF3/WT, SOD1(G93A), and ATF3/SOD1(G93A) mice at 90 d of age were subjected to RNA extraction using the TRIzol method (Invitrogen) with subsequent RNA cleanup and DNA digestion using the RNA Easy Micro Kit (Qiagen). RNA quality was tested using the 2100 Agilent Bioanalyzer (Agilent Technologies). The RNA was then reverse transcribed and labeled with biotin to generate biotin-labeled cRNA. Samples were then hybridized onto Illumina-Mouse WG6 v2.0 BeadChips. Arrays were then washed and stained with cy3-streapavidin and scanned in the Illumina BeadArray Reader. All 18 samples were processed at the same time under the same conditions. Each RNA sample used was extracted from one individual mouse. Five samples each were used for the SOD1(G93A) and ATF3/SOD1(G93A) mice, and four RNA samples each were used for the WT and ATF3/WT mice.

All analyses of expression were undertaken using the R programming environment (http://www.r-project.org). Probe summary profiles output by Illumina BeadStudio software after quantile normalization was read into R. Probes were retained for additional analysis only when the detection value was above 0.9 in at least two replicates from any of the tested conditions. Detection value was computed as one minus the detection P value characterizing the chance that the target sequence signal was distinguishable from the negative controls. Differential expression analysis was conducted by fitting a linear model to
18 microarrays and comparing the ATF3 transgenic genotypes with corresponding control using empirical Bayes moderated *t* statistics from the Bioconductor software package limma (59). For gene ontology (GO) analysis, the likelihood of over-representation of GO categories in the up- or down-regulated genes relative to the background of all array genes was calculated using the National Institute of Health’s DAVID Tool (Database for Annotation, Visualization, and Integrated Discovery), which implements a variant of Fisher exact test to test for enrichment of GO terms or Kyoto encyclopedia of genes and genomes (KEGG) pathways in a list of genes (60).


The Gene Set Enrichment Analysis (GSEA) (46) was implemented to effectively evaluate the effect of a specific experimental condition on known biological pathways and functional categories. The GSEA analysis was performed with GSEA v2.0 software (http://www.broadinstitute.org/gsea/software/software_index.html). Briefly, ranked expression lists were derived based on limma B score when the experimental conditions in question were compared with its corresponding control. An enrichment score is then calculated for an a priori gene list or gene set that is associated with a particular molecular classification. In this study, analysis was performed against the entire GSEA database (MSigDB September 2010; http://www.broadinstitute.org/gsea/msigdb/index.jsp) after filtering out gene sets smaller than 15 and larger than 500 genes (as recommended); 4,000 permutations were performed for each sample set. Finally, the enrichment scores are normalized to account for differences in gene set size, and false discovery rate (FDR) is then calculated relative to the normalized enrichment score values to determine the false-positive rate. Gene sets with significant FDR and P value were submitted to a leading edge analysis. Significant FDRs and P values were less than 25% and 0.001, respectively, in accordance with GSEA recommendations. Leading edge analysis extracts the genes that contributed most significantly to the enrichment score.
Fig. S1. ATF3 and NeuN immunostaining in the ventral horn of SOD1<sup>G93A</sup> and ATF3/SOD1<sup>G93A</sup> mice. Green, NeuN staining; red, ATF3 staining.
Fig. 52. Acetylcholine receptors and neurofilament double staining in the gastrocnemius muscle of SOD1<sup>G93A</sup> and ATF3/SOD1<sup>G93A</sup> mice. Green, α-bungarotoxin; red, neurofilament.
Forced ATF3 expression in SOD1 mutant mice alters the transcriptome. (A) Microarray analysis was performed on ventral horn samples of 90-d-old SOD1\textsuperscript{G93A}, ATF3/SOD1\textsuperscript{G93A}, ATF3/WT, and WT mice, with \( n = 5 \) for SOD1\textsuperscript{G93A} and ATF3/SOD1\textsuperscript{G93A} samples and \( n = 4 \) for ATF3/WT and WT samples. A heat map for all genes in the dataset relative to WT mice with a minimum cutoff change fold of 1.2 and \( P \) value \( \leq 0.05 \) comparing G93A, ATF3/G93A, and ATF3/WT is presented. (B) The Venn diagram illustrates the expression overlap of all sets of genes up- or down-regulated with a minimum cutoff change fold of 1.2 and \( P \) value \( \leq 0.05 \) relative to WT. (C and D) Pathways that are significantly down-regulated in the ATF3/SOD1\textsuperscript{G93A} compared with the SOD1\textsuperscript{G93A} mice as identified by GO analysis. (D) Pathways significantly up-regulated in the ATF3/SOD1\textsuperscript{G93A} compared with the SOD1\textsuperscript{G93A} mice as identified by GO analysis. (C and D) \_down represents a down-regulated pathway in each comparison, and \_up represents an up-regulated pathway in each comparison. Significance is presented as \( -\log(P \text{ value}) \), with a \( P \) value \( \leq 0.05 \) [that is, a \( -\log(P \text{ value}) \geq 1.3 \)] considered significant.
Fig. S4. Gene expression analysis reveals gene pathways involved in ATF3-mediated protection in ALS. (A–F) Heat maps showing the change fold difference in expression relative to WT for SOD1\textsuperscript{G93A} (G93A), ATF3/SOD1\textsuperscript{G93A} (ATF3/G93A), and ATF3/WT. [Scale bar: Log\textsubscript{2} (change fold relative to WT).] Green, down-regulated; red, up-regulated. (A) GO:0048699 ∼ generation of neurons. (B) GO:0019226 ∼ transmission of nerve impulse. (C) GO:0007010 ∼ cytoskeleton organization. (D) GO:0006917 ∼ induction of apoptosis. (E) GO:0051493 ∼ regulation of cytoskeleton. (F) GO:0007015 ∼ actin filament organization. (G) GSEA analysis using MSigDB (molecular signature database) showed that genes that are down-regulated in the aging human brain are up-regulated in ATF3/G93A compared with G93A mice. Genes are ranked by signal-to-noise ratio according to their differential expression between ATF3/G93A and G93A. Genes in the gene set down-regulated in aging human brain are marked with vertical bars, and the enrichment score is shown in green. This gene set is highly modulated by ATF3, with a nominal P value ≤ 0.0001 and an FDR = 0.059. (H) Genes comprising the leading edge of the GSEA aging brain dataset are presented in a heat map. [Scale bar: Log\textsubscript{2} (change fold relative to WT control).]
Fig. S5. Gene pathways involved in ATF3-mediated protection and pathways that are uniquely up-regulated in ATF3/SOD1(G93A). (A) Several immune and inflammatory response pathways detected by GO analysis were significantly down-regulated in ATF3/G93A compared with G93A mice. The pathways were scanned for redundancy and then combined into one heat map termed neuroinflammation (more information in Materials and Methods). Heat maps show the change fold difference in expression relative to WT for SOD1(G93A), ATF3/SOD1(G93A), and ATF3/WT. [Scale bar: Log$_2$(change fold relative to WT).] Green, down-regulated; red, up-regulated. (B) GO:0007399—nervous system development. (C) GO:0008543—FGF receptor (FGFR) signaling pathway. (D) GSEA analysis detected a significant difference in the HSA04360 axon guidance pathway in ATF3/G93A compared with G93A, with a nominal $P$ value of 0.0129 and FDR of 0.247. Genes in the leading edge include Ephb1, Chp, Dpys15, Pak3, Sema5a, Sema6a, Nfat5, Limk1, Pak6, Epha6, Cfl2, Epna5, Rgs3, Efnb3, Srgap2, Dpys12, Sema3a, Ab11, Pak1, Hras, Ppp3ca, Ppp3r3, Cxcl12, Srgap3, Plxna2, L1cam, Sema6d, Ntng1, Epha4, Epha6, Unc5b, Abilm3, Sema4f, and Mapk3. (E) The Venn diagram illustrates the expression overlap of all sets of genes that are up-regulated, with a minimum cutoff change fold of 1.2 and $P$ value $\leq 0.05$ relative to WT. (F) Pathways identified by GO analysis in the set of 74 genes that are uniquely up-regulated in ATF3/SOD1(G93A) compared with G93A. Significance is presented as $-\log(P$ value), with a $P$ value $\leq 0.05$ [that is, a $-\log(P$ value) $\geq 1.3$] considered significant. No significant pathways were identified in the same category of uniquely down-regulated genes.
Movie S1. Limb motion in SOD1G93A compared with ATF3/SOD1G93A at 132 d of age. Gender- and age-matched SOD1G93A and ATF3/SOD1G93A littermates (females) are displayed. The SOD1G93A littermate exhibits manifestations of paralysis, with visible decrease in limb motion, gait impairment, and dragging of rear quarters. In contrast, its ATF3/SOD1G93A littermate preserves full range of motion.

Movie S1

Movie S2. End stage in the SOD1G93A littermate. The same littermates shown in Movie S1 are presented at 137 d of age. The SOD1G93A littermate reached end stage, whereas the ATF3/SOD1G93A littermate is viable and only begun to display some limb motion impairment. This ATF3/SOD1G93A littermate reached end stage 14 d later at 151 d of age.

Movie S2