

Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by a selective degeneration of motor neurons, atrophy, and paralysis of skeletal muscle. Although a significant proportion of familial ALS results from a toxic gain of function associated with dominant SOD1 mutations, the etiology of the disease and its specific cellular origins have remained difficult to define. Here, we show that muscle-restricted expression of a localized insulin-like growth factor (Igf) -1 isoform maintained muscle integrity and enhanced satellite cell activity in SOD1^{G93A}

transgenic mice, inducing calcineurin-mediated regenerative pathways. Muscle-specific expression of local Igf-1 (mlgf-1) isoform also stabilized neuromuscular junctions, reduced inflammation in the spinal cord, and enhanced motor neuronal survival in SOD1^{G93A} mice, delaying the onset and progression of the disease. These studies establish skeletal muscle as a primary target for the dominant action of inherited SOD1 mutation and suggest that muscle fibers provide appropriate factors, such as mlgf-1, for neuron survival.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disorder involving the degeneration of motor neurons accompanied by skeletal muscle atrophy and paralysis. Transgenic mice ubiquitously overexpressing human SOD1 mutants develop motor neuron disease resembling ALS (Gurney et al., 1994) and provide useful model to test potential therapeutic strategies in disease treatment.

Notably, restriction of SOD1 mutant expression selectively to post-natal motor neurons failed to produce detectable sign of pathology or motor-neuron disease (Lino et al., 2002), suggesting that other cell types may be involved in ALS-associated neurodegeneration.

Indeed, analysis of chimeras generated between wild-type and SOD1 mutant mouse embryonic cells revealed that wild-type non neuronal cells in adult chimeric animals extended the survival of SOD1 mutant motor neurons, suggesting that the neurodegenerative action of mutant SOD1 may operate

through a dominant paracrine activity emanating from non-neuronal cells (Clement et al., 2003).

Skeletal muscle is an untested component in the motor neurodegenerative effects of SOD1 mutations. Adult muscle fibers are a source of signals that influence neuron survival, axonal growth, and maintenance of synaptic connections (Funakoshi et al., 1995). Among these, insulin-like growth factor 1 (Igf-1) has been implicated in anabolism of muscle and nerve tissues, inducing muscle hypertrophy and promoting neuronal survival (Musarò and Rosenthal, 2002).

In a recent study, injection of SOD1 mutant mouse muscle with an adeno-associated virus carrying an Igf-1 gene prolonged life and delayed disease progression (Kaspar et al., 2003). Retrograde transport of the AAV-Igf-1 vector to the spinal cord was reported necessary to achieve therapeutic effects (Kaspar et al., 2003). However, it is not clear from that study which Igf-1 isoform was used, or whether the effects of Igf-1 expressed in motor neurons were cell autonomous.

Two major isoforms of Igf-1, originating from alternative splicing, have been described, differing in structure and functions (Musarò and Rosenthal, 2002). These isoforms are referred as circulating (class 2) and local (class 1) Igf-1. Igf-1 class 2 transcripts predominate in the liver, are highly growth hormone (GH) responsive and as such are major endocrine effectors of

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Abbreviations used in this paper: AChR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; CnA, calcineurin; GFAP, glial fibrillary acidic protein; Igf, insulin-like growth factor; mlgf-1, local isoform of Igf-1; MyHC, myosin heavy chain; SOD1, superoxide dismutase1; wt, wild-type.

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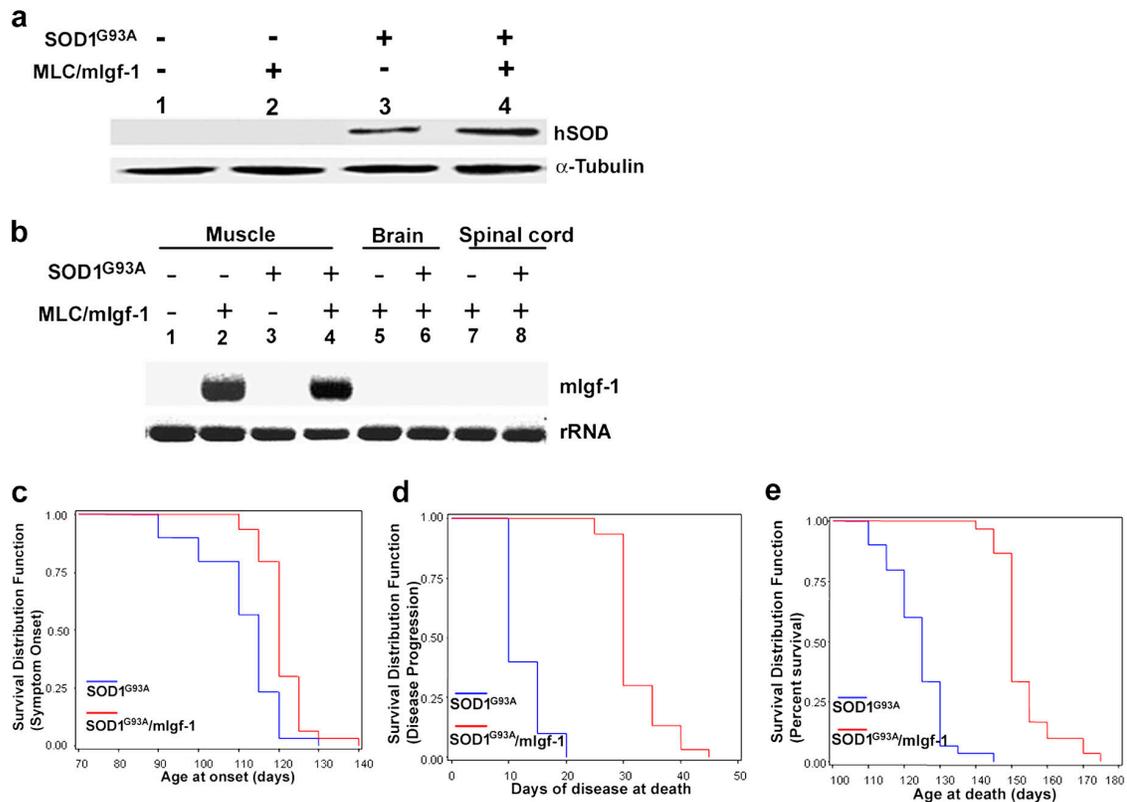


Figure 1. **mlgf-1** expression delays the progression of the disease and enhances the survival of SOD1^{G93A} mutant mice. (a) Western blot analysis of human SOD transgenic protein in wild-type (lane 1), MLC/mlgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mlgf-1 (lane 4) transgenic muscle. (b) Northern blot analysis for mlgf-1 transgene expression in skeletal muscle of wild-type (wt; lane 1), MLC/mlgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mlgf-1 (lane 4) transgenic mice; in brain and spinal cord of MLC/mlgf-1 (lanes 5 and 7) and SOD1^{G93A}/mlgf-1 (lanes 6 and 8) mice. (c) Age of onset of disease symptoms; average onset: SOD1^{G93A} ($n = 30$) = 111 ± 1.8 ; SOD1^{G93A}/mlgf-1 ($n = 30$) = 120.8 ± 1.0 (d) analysis of the progression of the disease; average of disease duration: SOD1^{G93A} ($n = 30$) = 12 ± 0.6 ; SOD1^{G93A}/mlgf-1 ($n = 30$) = 32 ± 0.8 (e) survival analysis; average survival: SOD1^{G93A} ($n = 30$) = 123 ± 1.4 ; SOD1^{G93A}/mlgf-1 ($n = 30$) = 152.8 ± 1.4 .

GH. By contrast, class 1 transcripts are widely expressed in all tissues, but remain preferentially confined to the tissue of expression. The contribution to more localized accumulation of Igf-1 class 1 seems due to the combination of exon 1, Ea peptide, and a lower binding affinity to Igf binding proteins, compared with circulating class 2 isoform (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200407021/DC1>).

To assess the effects of supplemental Igf-1 directly on atrophic SOD1 skeletal muscle, we exploited a transgenic mouse expressing a full-length precursor of the local isoform of Igf-1 (mIgf-1) that is normally induced transiently in response to muscle damage, but does not enter the circulation (Musarò et al., 2001; Musarò and Rosenthal, 2002). Muscle restricted mIgf-1 transgene (MLC/mIgf-1) exerts its effects in an autocrine/paracrine manner, circumventing the adverse side effects of systemic Igf-1 administration. Expression of the MLC/mIgf-1, delivered either as an inherited transgene or somatically on an AAV vector, induces muscle hypertrophy and strength, and preserves regenerative capacity in senescent and dystrophic mice (Barton-Davis et al., 1998; Musarò et al., 2001; Barton et al., 2002) through enhanced stem cell recruitment (Musarò et al., 2004).

In the present study, we establish skeletal muscle as a primary target in inherited forms of ALS by showing that localized expression of the coinherited MLC/mIgf-1 transgene

exclusively in the skeletal muscle of SOD1^{G93A} mice counteracted the symptoms of ALS, induced satellite cell activity, stabilized neuromuscular junctions, and led to a reduction in astrocytosis in the SOD1^{G93A} spinal cord. These observations suggest novel approaches to the attenuation of motor neuronal degradation and underscore the importance of Igf-1 isoform selection when designing therapeutic strategies for ALS.

Results and discussion

Muscle-specific mIgf-1 delays the progression of the disease and prolongs the life span of SOD1^{G93A} mice

To evaluate the effects of mIgf-1 on the SOD1^{G93A} neurodegenerative phenotype, we compared double transgenic SOD1^{G93A} and MLC/mIgf-1 transgenic mice to their SOD1^{G93A} littermates. The SOD1^{G93A} and SOD1^{G93A} × MLC/mIgf-1 (SOD1^{G93A}/mlgf-1) transgenic mice were selected for same expression level of the human transgenic protein (Fig. 1 a). Notably, the mIgf-1 transgene was selectively expressed in skeletal muscle of both MLC/mIgf-1 and SOD1^{G93A}/mlgf-1 mice (Fig. 1 b, lanes 2 and 4), whereas it was not expressed in the brain and spinal cord (Fig. 1 b, lanes 5–8), or in skeletal muscle of wild-type and SOD1^{G93A} mice (Fig. 1 b, lanes 1 and 3).

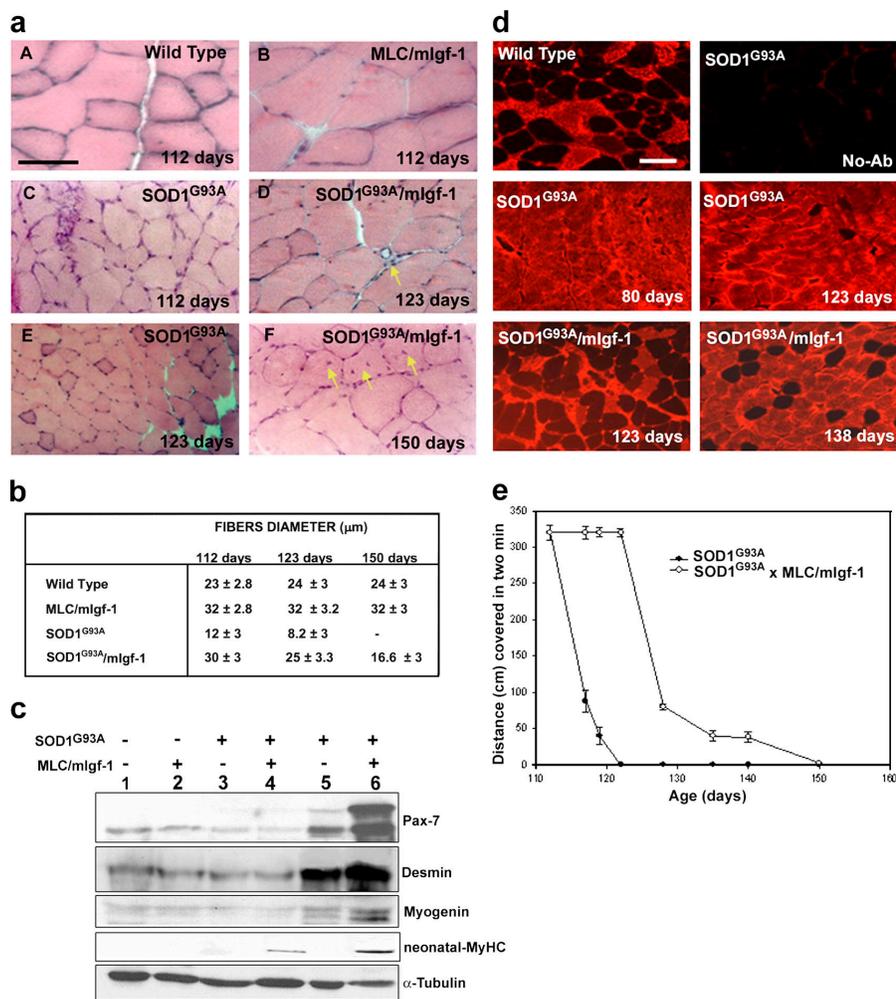


Figure 2. mlgf-1 expression attenuates muscle wasting and promotes regenerative pathways in SOD1^{G93A} mice. (a) Histological analysis of wt, MLC/mlgf-1, SOD1^{G93A} and SOD1^{G93A}/mlgf-1 muscle at different age and stage of disease. Bar, 20 μm (b) Analysis of fiber size differences in the quadriceps underscores the relative attenuation of muscle atrophy in SOD1^{G93A}/mlgf-1 compared with SOD1^{G93A} mice. (c) Western blot analysis of molecular markers of muscle regeneration, activated satellite cells, and maturation. Muscle protein lysates were obtained from quadriceps of wt (lane 1), MLC/mlgf-1 (lane 2), SOD1^{G93A} (lanes 3 and 5) and SOD1^{G93A}/mlgf-1 (lanes 4 and 6) transgenic mice at different ages of the clinical disease (lanes 3 and 4 at 28 d old; lanes 5 and 6 at 123 d old). Immunoblotting for α -tubulin served as a control for protein loading. (d) Immunofluorescence analysis of MyHC-fast performed on soleus muscles of wt, SOD1^{G93A} and SOD1^{G93A}/mlgf-1 before (80 d) and after symptom onset (123, 138 d old). Bar, 50 μm . (e) Walk test of SOD1^{G93A} (closed circles) and SOD1^{G93A}/mlgf-1 (open circles) transgenic mice.

At 111 ± 1.8 d old, disease onset was observed in the mutant SOD1^{G93A} transgenic mice ($n = 30$; Fig. 1 c). Notably, the SOD1^{G93A} mice died within $10 \text{ d} \pm 0.6$ of clinical disease onset (Fig. 1 d), with a maximal life span of 145 d (Fig. 1 e). By contrast, localized expression of mlgf-1 delayed the onset (Fig. 1 c) and in particular the progression (Fig. 1 d) of disease, increasing the survival of SOD1^{G93A}/mIgf-1 mice ($n = 30$) by ~ 30 d to a maximal life span of 175 d (Fig. 1 e). Differences between SOD1^{G93A} and SOD1^{G93A}/mIgf-1 were significantly relevant for onset ($\chi^2_{\text{LR}} = 18.7$, $P < 0.0001$), progression ($\chi^2_{\text{LR}} = 67.0$, $P < 0.0001$), and survival ($\chi^2_{\text{LR}} = 63.2$, $P < 0.0001$).

mlgf-1 expression attenuates muscle atrophy, increasing satellite cell activation in SOD1^{G93A} mice

SOD1^{G93A} ($n = 7$) and SOD1^{G93A}/mIgf-1 ($n = 7$) transgenic mice were analyzed at different stages of disease. At 123 d, motor neuronal degeneration of SOD1^{G93A} mice was accompanied by severe muscle atrophy (Fig. 2 a, E), and complete muscle paralysis (hereafter this stage is indicated as paralysis stage). In contrast, at the same age, SOD1^{G93A}/mIgf-1 transgenic mice did not show evident signs of muscle disease, but only difficulty in extending the limbs when suspended (hereafter this stage is indicated as symptom onset). Moreover, muscle

atrophy was substantially attenuated in SOD1^{G93A}/mIgf-1 offspring even after onset of denervation and paralysis stage (153 d old; Fig. 2 a, F; Fig. 2 b). In addition, markers of satellite cell activity, such as Pax-7 and desmin, were increased to varying extents in affected SOD1^{G93A} mice (Fig. 2 c), whereas hallmarks of satellite cell activity and fiber maturation, including centralized nuclei (Fig. 2 a, yellow arrows), Pax-7 isoforms, desmin, myogenin, and neonatal myosin heavy chain (MyHC) expression were present exclusively in the SOD1^{G93A}/mIgf-1 muscles at all stages of disease, including at paralysis stage (Fig. 2 c and not depicted). Thus, satellite cell activation is likely to contribute to the maintenance of muscle phenotype induced by mIgf-1 expression (Barton-Davis et al., 1999).

Motor neurons are known to regulate the properties of the myofibers they innervate by selective activation of fiber specific gene expression. Immunohistochemical analysis (Fig. 2 d) revealed that fiber type composition was altered in SOD1^{G93A} soleus muscle even before overt disease (80 d), with a shift toward a fast fiber type. In contrast, normal heterogeneity of muscle fibers was maintained for a more extended period in SOD1^{G93A}/mIgf-1 mice, which showed shifts in fiber composition only at later stage of disease (Fig. 2 d, 138 d). By paralysis stage (153 d) there was not significant difference in fiber type composition between SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice

(not depicted). The alteration in the heterogeneity of SOD1^{G93A} muscle fibers indicate an alteration in motor neuron activity even before overt disease and support the hypothesis that delaying the progression and severity of ALS disease may depend on the maintenance of muscle integrity.

The walk test analysis (Fig. 2 e), performed at different ages, revealed that at 112 d SOD1^{G93A} mice ($n = 7$) showed symptom onset without evident alterations in functional parameters. The condition of SOD1^{G93A} mice rapidly deteriorated at 117 d, as shown by the shortening of their walk (Fig. 2 e). In contrast, the pathological sign of disease were delayed in SOD1^{G93A}/mIgf-1 transgenic mice ($n = 7$), as shown by the capacity of the mice to walk 233 ± 5.6 cm further when analyzed at same age as SOD1^{G93A} mice, and by their ability to move for a more extended period of time (Fig. 2 e).

These data suggest that promotion of muscle satellite cell activity and hypertrophy through mIgf-1 can be considered as an alternate therapeutic approach to counteract muscle wasting associated with ALS disease.

An activated calcineurin isoform is induced in SOD1^{G93A}/mIgf-1 muscle

One of the signal transduction pathway involved in muscle remodeling is the protein phosphatase calcineurin-A (CnA). CnA overexpression has been implicated in myocyte hypertrophy (Musarò et al., 1999), fiber type conversion (Chin et al., 1998), and tissue remodeling after injury (Sakuma et al., 2003). In a study of selective CnA subunit isoform expression, we recently established that the alternatively spliced variant CnA- β 1 is up-regulated in regenerating skeletal muscle fibers (unpublished data).

In this study, we verified whether activation of satellite cells and maintenance of muscle phenotype involved the induction of CnA- β 1 expression. Although the normally low levels of CnA- β 1 expression were not raised in SOD1^{G93A} muscles (Fig. 3 a lane 3; Fig. 3 b lane 5, Fig. 3 c), or in uninjured wild-type and MLC/mIgf-1 muscle (Fig. 3, a and b, lanes 1 and 2), SOD1^{G93A}/mIgf-1 regenerating muscle dramatically increased CnA- β 1 transcripts ($73 \pm 1.3\%$; Fig. 3 a lane 4) and protein ($52 \pm 2\%$; Fig. 3 b lane 6, Fig. 3 c) after onset of clinical symptoms. Thus, specific signaling elicited by CnA- β 1 may contribute to the prolongation of muscle integrity even after denervation.

Preservation of neuromuscular junctions in SOD1^{G93A}/mIgf-1 mice

Alterations in motor neuronal activity typical of denervated muscle and motor neuron diseases also affect the configuration of neuromuscular junctions in SOD1^{G93A} mice, characterized by the diffusion of acetylcholine receptor (AChR) postsynaptic clusters (Fig. 4 a, yellow arrow). At 123 d, SOD1^{G93A} paralyzed muscle showed $56 \pm 0.2\%$ of diffuse AChR expression, whereas AChR cluster aggregates (Fig. 4 a) were preserved in muscles of age-matched SOD1^{G93A}/mIgf-1 mice, which showed only $3.3 \pm 0.4\%$ of diffuse AChR expression. At comparable end-stage disease, SOD1^{G93A}/mIgf-1 muscle displayed only $18 \pm 0.4\%$ of diffuse AChR expression. These results were confirmed by Northern blot analysis (Fig. 4 b); high

AChR expression levels in SOD1^{G93A} muscle were reduced in SOD1^{G93A}/mIgf-1 mice at all stages observed. Densitometric analysis ($n = 6$) revealed that AChR mRNA expression in SOD1^{G93A} paralyzed muscle (123 d) was $68 \pm 2.4\%$ higher than that observed in age matched SOD1^{G93A}/mIgf-1 mice; whereas the increase in mRNA expression in SOD1^{G93A} mice was of $32 \pm 1.9\%$ when SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice were analyzed at comparable end-stage disease. This suggests that mIgf-1 delays the progression of the disease, stabilizing the innervation of muscle fibers.

The localization of AChR clusters at the end plate requires the expression of agrin, a large proteoglycan in the synaptic cleft that plays an important role in the maintenance of the molecular architecture of the postsynaptic membrane (McConville and Vincent, 2002). Agrin expression was significantly down-regulated in paralyzed SOD1^{G93A} compared with SOD1^{G93A}/mIgf-1 muscle (Fig. 4 c) analyzed at comparable end-

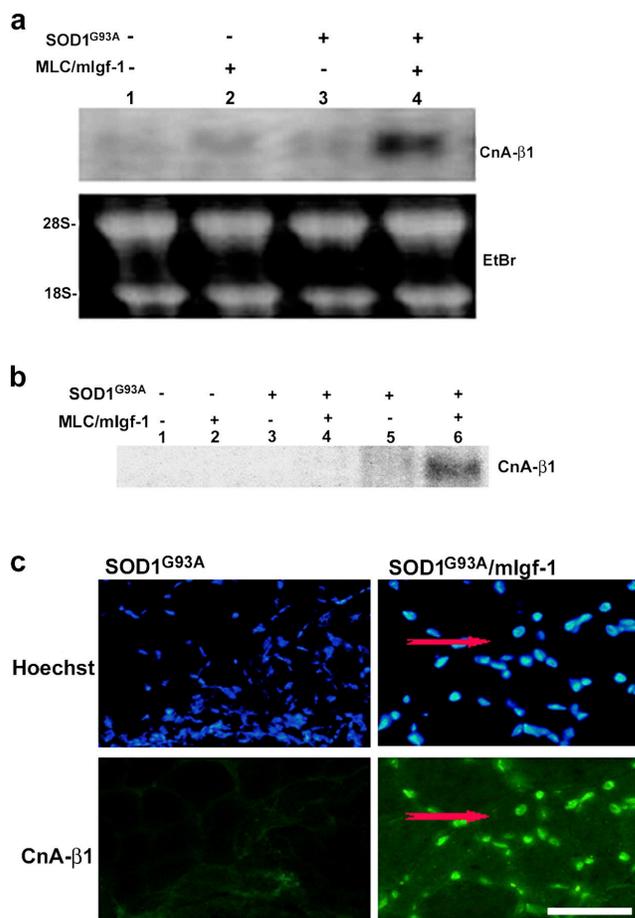


Figure 3. Transgenic mIgf-1 expression induces chronic CnA- β 1 expression in SOD1^{G93A} mice. (a) Northern blot analysis of CnA- β 1 expression in wt (lane 1), MLC/mIgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mIgf-1 (lane 4) transgenic mice. Ethidium bromide staining was used to verify equal loading of the RNA sample. (b) Lysates of the same muscle tissues used in Fig. 2 c were tested by Western blotting using a CnA- β 1-specific antibody. (c) Immunofluorescence of transverse sections from quadriceps muscles of SOD1^{G93A} and SOD1^{G93A}/mIgf-1 at paralysis stage. CnA- β 1 shows a nuclear localization. A regenerating fiber is indicated by the presence of central nucleus (red arrows). Nuclei were visualized by Hoechst dye (blue). Bar, 20 μ m.

stage disease, further underscoring a role for local expression of mIgf-1 in the maintenance of muscle innervation.

Muscle-restricted mIgf-1 prolongs motor neuronal function in SOD1^{G93A} mice

Histological analysis of the ventral spinal cord revealed that SOD1^{G93A} mice ($n = 7$) presented a progressive reduction in the number of motor neuron from clinical onset to end-stage disease. Specifically, SOD1^{G93A} mice showed a reduction of 37 and 55% in the number of motor neurons at clinical onset (112 d) and at end-stage disease (123 d), respectively (Fig. 5 a). In contrast, mIgf-1 expression induced motor neuron survival in SOD1^{G93A}/mIgf-1 mice ($n = 7$) at all ages and stages observed, with significant differences at 112 and 123 d old (Fig. 5 a), demonstrating that mIgf-1 expression preserves the motor neuron during the progression of ALS.

One of the prominent markers of motor neuron dysfunction in ALS mice is the activation of astrocytes, leading to motor weakness and neural loss (Hall et al., 1998). Comparable patterns of glial fibrillary acidic protein (GFAP) immunoreac-

tivity were found in spinal cords of SOD1^{G93A} ($n = 6$) and SOD1^{G93A}/mIgf-1 ($n = 6$) transgenic mice before the symptom onset (28 d; Fig. 5 b, A, B, and D, insert, lanes 1 and 2). However, at paralysis stage (123 d) the spinal cord of SOD1^{G93A} mice demonstrated a marked increase in astroglial activation (Fig. 5 b C), with an increase in GFAP expression of $\sim 55 \pm 0.2\%$ (Fig. 5 b, D, insert, lane 3) compared with the GFAP expression levels displayed in the spinal cord of age matched SOD1^{G93A}/mIgf-1 transgenic mice (Fig. 5 b, D and insert, lane 4). At comparable end-stage disease, there were no significant differences in GFAP expression between SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice, although SOD1^{G93A} mice continued to express 13% more GFAP as compared with SOD1^{G93A}/mIgf-1 mice (unpublished data).

The activation of astroglia can be correlated with the expression of certain cytokines, such as TNF- α , which enhance

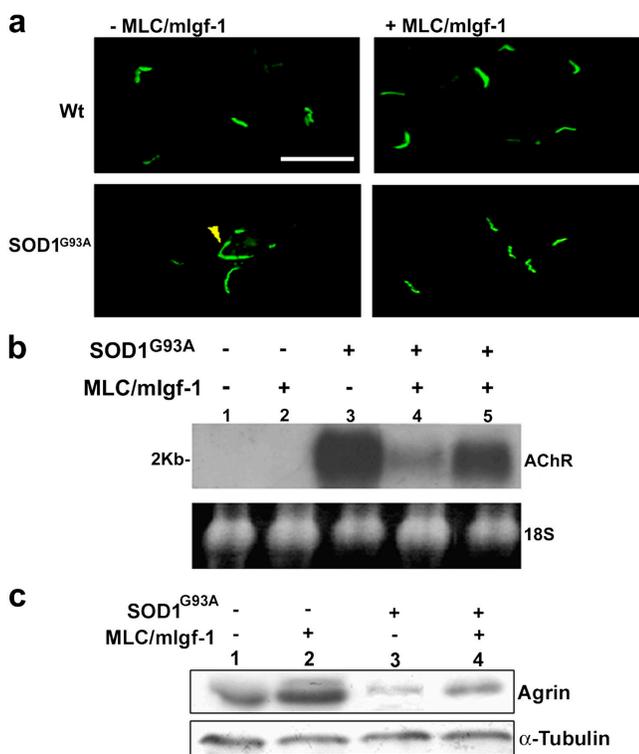


Figure 4. Maintenance of the neuromuscular junction configuration in SOD1^{G93A}/mIgf-1 transgenic mice. (a) Immunofluorescent analysis of transverse sections from muscles of wt, MLC/mIgf-1, SOD1^{G93A} and SOD1^{G93A}/mIgf-1 transgenic mice at 123 d old. α -bungarotoxin antibody identified diffusion of AChR expression in SOD1^{G93A} muscle (yellow arrow); whereas SOD1^{G93A}/mIgf-1 muscle maintained AChR clusters. Bar, 20 μ m. (b) Northern blot of total RNA samples from quadriceps of wt (lane 1), MLC/mIgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mIgf-1 (lanes 4 and 5) transgenic muscles at 123 (lanes 1–4) and 150 (lane 5) days old, hybridized with AChR ³²P-labeled probe. Ethidium bromide staining was used to verify equal loading of the RNA sample. (c) Western blot of agrin from quadriceps of wt (lane 1), MLC/mIgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mIgf-1 (lane 4) transgenic muscles. SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice were analyzed at comparable end-stage disease.

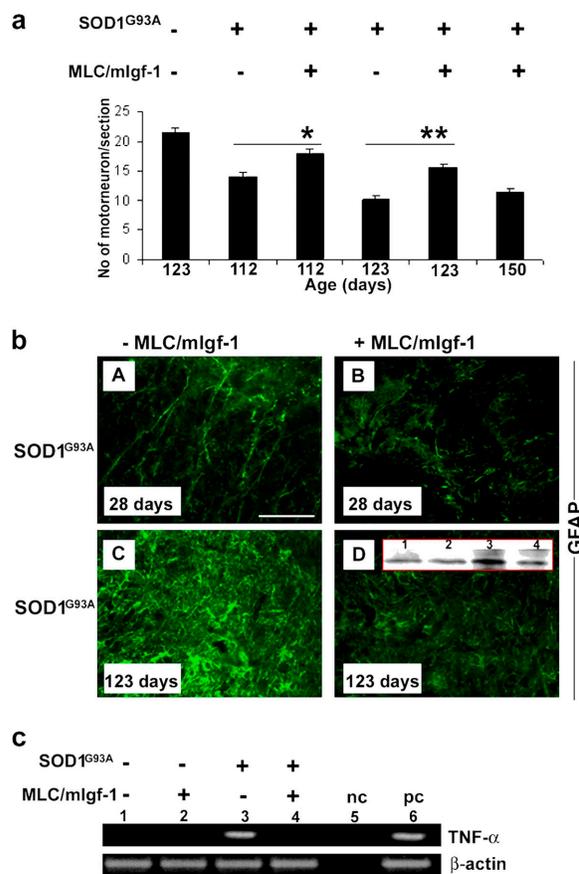


Figure 5. Transgenic mIgf-1 expression protects motor neuron from degeneration. (a) Quantification of surviving motor neurons in the ventral spinal cord of wild-type, SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice at different ages. * $P < 0.001$; ** $P < 0.001$. (b) Immunofluorescence analysis identify GFAP positive astrocytes in ventral horn of SOD1^{G93A} and SOD1^{G93A}/mIgf-1 mice at different ages: A and B, 28 d old; C and D, 123 d old. The intensity of the GFAP signal revealed progressive astrocytosis. Bar, 20 μ m. Insert in D shows Western blot for GFAP in the spinal cord of SOD1^{G93A} (lanes 1 and 3) and SOD1^{G93A}/mIgf-1 (lanes 2 and 4) mice at 28 (lanes 1 and 2) and 123 (lanes 3 and 4) d old. (c) RT-PCR analysis of TNF- α and β -actin of wt (lane 1), MLC/mIgf-1 (lane 2), SOD1^{G93A} (lane 3) and SOD1^{G93A}/mIgf-1 (lane 4) transgenic mice at 123 d old. Lane 5 shows a negative control (nc) consisting of RT-PCR mix without cDNA template. Lane 6 identifies the RNA positive control (pc) for TNF- α obtained from spleen.

the response to inflammatory states and contribute to the progression of neurological dysfunction in SOD1^{G93A} mice (Elliott, 2001). Although TNF- α expression was normally undetectable in the CNS of healthy mice (Fig. 5 c, lanes 1 and 2), it accumulated in the spinal cord of SOD1^{G93A} mice at paralysis stage (123 d; Fig. 5 c, lane 3). In contrast, TNF- α expression was not apparent in the spinal cord of SOD1^{G93A}/mIgf-1 transgenic mice (Fig. 5 c, lane 4). mIgf-1 hypertrophic muscle therefore functions as a protective tissue for the CNS, modulating reactive astrogliosis and inflammatory cytokines that normally exacerbate the pathogenesis of ALS disease.

ALS is emerging as a "multi-systemic" disease in which the alteration in structural, physiological, and metabolic parameters in different cell types (muscle, motor neuron, glia) may act synergistically to exacerbate the disease and evidences a functional cross-talk between neuronal and non-neuronal cells (Rothstein et al., 1996; Ferri et al., 2004). The present study serves to refocus therapeutic strategies to attenuate motor neuronal degradation toward skeletal muscle. It remains to be determined whether the dramatic prolongation of CNS tissue integrity in SOD1^{G93A}/mIgf-1 mice derives from the direct retrograde transport of transgenic mIgf-1, or indirect action either through distal activation of endogenous Igf-1 expression, or through other trophic factors secreted by SOD1^{G93A}/mIgf-1 muscle.

In a previous study, retrograde transport of the AAV-Igf-1 vector from muscle to motor neuron was deemed necessary to achieve the therapeutic effects, because the same gene delivered to the muscle by a lentiviral vector, which cannot retrotransport, proved ineffective.

The results of the present study suggest other explanations. The Igf-1 cDNA used by Kaspar et al. (2003) may have encoded the more prevalent circulating gene product, which once secreted from the muscle disperses in the circulation (Musarò and Rosenthal, 2002). In contrast, the mIgf-1 isoform used in our present and previous studies (Barton-Davis et al., 1998; Musarò et al., 2001; Barton et al., 2002; Musarò et al., 2004) does not enter the circulation, but accumulates in the tissue of synthesis where its autocrine/paracrine function is concentrated. The importance of Igf-1 isoform choice in designing therapeutic strategies cannot be overstressed, because their diverse biological activities lead to radically different outcomes (for review see Musarò and Rosenthal, 2002). Whatever its mode of action, the feasibility of localized synthesis of the mIgf-1 isoform to activate survival mechanisms in distal damaged tissues represents a powerful approach to counteract the degeneration of both muscle and motor neuron in ALS disease.

Materials and methods

Mice

SOD1^{G93A} transgenic mice (Jackson Laboratory) express a transgenic human mutant SOD1^{G93A} allele containing the Gly93 \rightarrow Ala (G93A) substitution, driven by its endogenous human promoter (Gurney et al., 1994). The SOD1^{G93A} B6J mice were crossed with MLC/mlgf-1 FVB mice (Musarò et al., 2001) for seven different generations to obtain SOD1^{G93A}/mlgf-1 B6J inbred transgenic mice.

Walk test

The walk test was performed in a scaled ramp, accordingly to the method reported by Gurney et al. (1994). The mice were allowed to explore the cage for 1 min and then they were left to walk for 2 min. The hind feet of the mice were painted with ink and the track left by the mice were recorded on a paper tape. The test was performed in horizontal, laminar flow hood to maintain barrier conditions.

Histological and immunofluorescence analysis

For histological analysis, 7- μ m tissue cryosections were fixed in 4% PFA and stained with hematoxylin/eosin. For immunofluorescence analysis, sections were fixed with 4% PFA, washed in PBS with 1% BSA and 0.2% Triton X-100, preincubated for 1 h in 10% goat serum at RT, and incubated overnight at 4°C with primary antibodies: neonatal MyHC (neo-MyHC), MyHC-fast, Alexa Fluor 488-conjugated α -bungarotoxin, CnA- β 1 (from C. Klee, National Institutes of Health, Bethesda, MD), GFAP. Nuclei were visualized by Hoechst staining.

For motor neuron quantification, fixed spinal cord slices were immunostained with SMI-32 monoclonal antibody

Inverted microscope (Axioskop 2 plus; Carl Zeiss MicroImaging, Inc.) using 20 or 40 \times lenses was used, and images were processed using Axiovision 3.1.

RNA preparation and Northern analysis

RNA was extracted from muscles by RNA-Trizol-kit (GIBCO BRL). Total RNA was separated in 1.3% agarose gel and hybridized as described previously (Musarò and Rosenthal, 1999).

RT-PCR analysis

RNA was isolated from spinal cord of wild-type, MLC/mlgf-1, and SOD1^{G93A} and SOD1^{G93A}/mlgf-1 transgenic mice. The following oligonucleotides were used: TNF- α sense, 5'-CCCAGACCCTCACACTCAGAT-3' and anti-sense, 5'-TTGTCCTTGAAGAGAACCTG-3'; β -actin sense, 5'-GTGGGCCGCTCTAGGCACAA-3' and anti-sense, 5'-CTCTTGTATGTCACGCACGATTTC-3'.

Protein extraction and Western blot analysis

Protein extraction was performed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% wt/vol Triton X-100, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM PMSF, 1 μ g/ml of each aprotinin, leupeptin, pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride). Equal amounts of protein from each muscle lysate were separated in SDS polyacrylamide gel and transferred onto a hybond C extra nitrocellulose membrane. Filters were blotted with antibodies against hSOD, Pax7, myogenin, desmin, neo-MyHC (from S. Schiaffino, University of Padova, Padova, Italy), Agrin, GFAP.

Online supplemental material

Organization of the Igf-1 gene. As its name implies, Igf-1 is similar to insulin in structure. The mature Igf-1 is a single-chain protein of 70 aa and differs from insulin by retention of the C-domain, by a short extension of the A-domain to include a novel domain D, and by the presence of variable COOH-terminal extension peptides (E-peptides; Fig. S1). The rodent Igf-1 gene contains six exons, separated by five introns (Fig. S1). Exons 1 and 2 encode distinct 5'UTRs, as well as different parts of the signal peptide, and are therefore termed leader exons. Separate promoters drive exons 1 and 2 transcription. Exon 3 encodes 27 aa that are part of the signal peptide and common to all isoforms, as well as part of the mature Igf-1 peptide. Exon 4 encodes the rest of the mature peptide and 16 aa of the NH₂-terminal region of the E-peptide, which is also common to all Igf-1 mRNAs. Exons 5 and 6 encode two distinct COOH-terminal E-peptides (Ea, Eb) and the 3'UTR. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200407021/DC1>.

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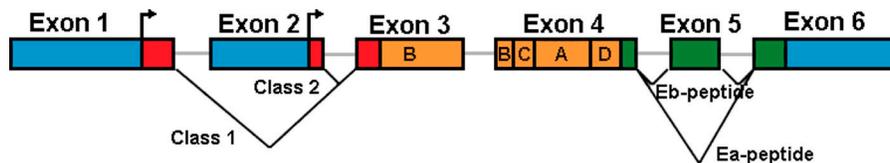


Figure S1. **Complexities of IGF-1 transcription: schematic representation of IGF-1.** The amino acid sequence of the mature IGF-1 peptide differs from that of insulin by retention of the C peptide, by a short extension of the A chain to include a novel domain D, and by the presence of variable COOH-terminal E peptides (Fig. S1). Two major isoforms of IGF-1, have been characterized, originating from alternative splicing and differing in structure and function. These isoforms are referred as circulating (class 2) and local (class 1) IGF-1.

The rodent IGF-1 gene contains six exons, separated by five introns (Fig. S1). Exons 1 and 2 encode distinct 5'UTRs (blue box), as well as different parts of the signal peptide (red box), and are therefore termed leader exons. Exon 3 encodes 27 aa that are part of the signal peptide (red box) and common to all isoforms, as well as part of the mature IGF-1 peptide (orange box). Exon 4 encodes the rest of the mature peptide (orange box) and 16 aa of the amino-terminal region of the E-peptide (green box), which is also common to all IGF-1 mRNAs. Exons 5 and 6 encode two distinct carboxy-terminal E-peptides (green box) and the 3'UTR (blue box). Separate promoters drive exon 1 and exon 2 transcription (Fig. S1, arrows).

Transcripts initiated at exon 1 are spliced to produce transcripts that encode exon 1 but lacks exon 2, whereas transcripts initiated at exon 2 give rise to transcripts that encode exon 2 but lacks exon 1. Although IGF-1 transcripts are not exclusively tissue-restricted, those that initiate at exon 2 (class 2) predominate in the liver, are highly growth hormone responsive and as such are major endocrine effectors of GH. By contrast, transcripts initiating at exon 1 (class 1) are widely expressed in all tissues, and are less affected by circulating growth hormone levels, presumably performing autocrine or paracrine functions. Indeed, a liver-specific knockout of the IGF-1 gene resulted in a 75% decrease in circulating IGF-1 levels, but did not affect the size of the animals (Yakar, S., J.L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer, and D. LeRoith. *Proc. Natl. Acad. Sci. USA.* 1999. 96:7324–7329), providing further evidence for a paracrine role of locally expressed IGF-1.

Elucidation of isoform function is also complicated by alternate splicing at the 3' end of IGF-1 transcripts. This produces variability in the length and amino acid sequence of the E peptide, and in the length and base sequence of the 3'UTR. To date, two different splice patterns have been documented in rodents. Each generates E peptides with a common NH₂-terminal 16 aa sequence, and alternate COOH-terminal sequences. If exon 4 splices to exon 6 (the predominant pattern), the length of the 3'UTR is highly variable, but in all cases the Ea peptide is generated with 19 additional amino acids. If exon 4 splices to exons 5 and 6, a variant known as Eb is encoded, which is frameshifted relative to exon 6 and therefore a different 25 aa sequence is added to the common 16 aa encoded by exon 4. In muscles subjected to damage or exercise the exon 4–5–6 splice variant is up-regulated and the isoform encoded by this mRNA has been called mechano growth factor (MGF). Although the signal peptide included in MGF has not been specified, MGF presumably corresponds to IGF-1Eb in the rat.

Although E peptide choice appears to be independent of promoter use, Eb-containing transcripts are more abundant in liver, whereas Ea-containing transcripts are widespread in extra-hepatic tissues, including muscle, and the combination of exon 1 and Ea peptide could give rise to more localized accumulation of IGF-1. In addition, the analysis of the amino acid structure of both E-peptides has revealed the presence of two N-linked glycosylation sites only in the Ea peptide, but not in the Eb peptide, suggesting that this post-translational modification is involved in a biological action of the IGF-1 isoform.

Additional differences between circulating and local IGF-1 have been proposed for binding affinity to IGF binding proteins (IGFBPs), thus restricting the action of the local form of IGF-1.

The predominant IGF-1 mRNA variant expressed in skeletal muscle is initiated at exon 1 (class 1) with an exon 4–6 spliced variant, and represents the isoform we used in the present study.

