Altered Expression of Myogenic Regulatory Factors in the Mouse Model of Amyotrophic Lateral Sclerosis

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Key Words

Amyotrophic lateral sclerosis · Motor neuron disease · Myogenic regulatory factor · Muscle · Denervation

Abstract

Background: In the superoxide dismutase 1 (SOD1)-G93A mouse model of amyotrophic lateral sclerosis (ALS), skeletal muscle is a key target of mutant SOD1 toxicity. However, the expression of factors that control the regenerative potential of the muscle is unknown in this model. **Objective:** To characterize the expression of satellite cell marker *Pax7* and myogenic regulatory factors (MRF) in skeletal muscle of SOD1-G93A mice at different stages of the disease. **Methods:** The expressions of *Pax7*, *Myod1*, *Myf5* and myogenin (*Myog*) were determined by quantitative real-time PCR and by Western blotting from the grouped gastrocnemius, quadriceps and soleus muscles of SOD1-G93A mice at presymptomatic, symptomatic and terminal stages of the disease, and from surgically denervated wild-type gastrocnemius muscles. **Results:** *Pax7* mRNA and MYF5 protein were upregulated in presymptomatic mice, coinciding with increased muscle damage marker *Rrad* and chemokine *Ccl5*. All MRF transcripts and most proteins (excluding MYOG) were increased, starting from 3 months of age, simultaneously with

increased expression of denervation marker *Chrna1*. However, in the terminal stage, no protein increase was evident for *Pax7* or any of the MRF despite the increased mRNA levels. The transcripts for chemokine *Ccl2* and chemokine receptor *Cxcr4* were increased starting from the onset of symptoms. **Conclusions:** The characterization of *Pax7* and MRF in SOD1-G93A mice reveals a progressive induction of the myogenic program at the RNA level, but a blunted protein level response at late stages of the disease. Altered posttranscriptional and posttranslational mechanisms likely to operate, as well as the potential role of chemokine signaling in mutant SOD1 muscle, are discussed.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic motor neuron (MN) disease characterized by progressive muscle weakness caused by gradual degeneration of upper and lower motor neurons [1]. Approximately 10% of ALS cases are familial (FALS), and one fifth of these are caused by mutations in the gene encoding antioxidant enzyme Cu/Zn superoxide dismutase 1 (SOD1) [2]. This suggests that oxidative stress is one of the key mecha-

nisms that underlie ALS pathogenesis. Transgenic mouse models of FALS have been instrumental in shedding light on pathophysiological and molecular aberrations in human ALS patients [3]. The most frequently used model of FALS is a ubiquitous overexpressor of human SOD1 that carries a glycine-to-alanine substitution at residue 93 (SOD1-G93A) [4] and results in toxic gain of SOD1 function [5]. Although the precise cellular mechanisms of mutant SOD1 (mSOD1) toxicity remain unknown, SOD1-G93A mice develop pathological and clinical features closely resembling human ALS [6]. The primary trigger for the pathogenic events is thought to be the interruption of the nerve connection at the neuromuscular junction (NMJ), which precedes axonal degeneration and death of MN cell bodies [7–10]. Debate still continues as to what extent the muscle denervation in ALS models is of neuronal origin: neuron-restricted expression of the mSOD1 does not replicate the hallmarks of the disease [11, 12], although this may depend on the mSOD1 expression level [13]. On the other hand, the non-cell-autonomous contribution to MN degeneration by non-neural cells (such as microglia, astrocytes and muscle) is evident [14], and suggests that alterations in multiple cell types act synergistically to exacerbate the disease.

Involvement of skeletal muscle in ALS pathophysiology was first inferred from early muscle hypermetabolism in mSOD1 mice and in human patients [15–17]. Experimentally induced muscle hypermetabolism dismantles NMJ in wild-type mice as well as it promotes disease progression in mSOD1 animals [18]. Additionally, a denervation-promoting factor, Nogo-A, is upregulated in ALS skeletal muscle [19, 20]. Recent studies have demonstrated that skeletal muscle-specific mSOD1 overexpression is sufficient to induce oxidative damage and trigger ALS symptoms [21] as well as MN degeneration [22]. Collectively, these studies suggest that early changes in ALS muscle may promote denervation.

Postmitotic skeletal muscle fibers show a great degree of plasticity in response to exercise, damage and disease [23]. The capacity for tissue repair derives largely from activation of satellite cells (SC), mononuclear stem cells that reside beneath the basal membrane of the mature muscle fiber [24, 25]. *Pax7* is a paired-box transcription factor essential for the survival, maintenance and myogenic potential of SC [26, 27]. Muscle growth and differentiation are regulated by a complex interaction between myogenic regulatory factors (MRF) such as *Myf5*, *Myod1* and myogenin (*Myog*), which specifically activate the production of key muscle genes [28]. Following muscle injury, quiescent *Pax7*-positive SC migrate to the site of

damage, upregulate the myogenic determinants *Myf5* and *Myod1* and reenter the cell cycle [29–31]. These activated myoblasts subsequently downregulate *Pax7* and induce *Myog* to initiate terminal differentiation. This is followed by fusion with other SC or with damaged myofibers, cell cycle exit and progressive loss of MRF expression.

Transcriptional induction of MRF upon surgical denervation is well described [32–36]. However, although the denervation of NMJ in ALS is evident, potential alterations in myogenic regulation in FALS models remain unexplored. Skeletal muscle tissue in mSOD1 mice is not only affected by denervation but also suffers from nerve-independent loss of muscle homeostasis [21, 37]. Muscle-autonomous mSOD1 toxicity may limit the capacity of mutant muscle to regenerate, and altered expression of SC markers (such as *Pax7*) or MRF could serve as early indicators of such a process. Muscle regeneration may also be affected by recruitment of muscle-resident or circulating myogenic progenitor cells other than SC [38–42]. Directional cell migration to muscle may be enhanced by the action of secreted chemotactic cytokines (chemokines), some of which are upregulated at least in the circulation and the cerebrospinal compartment of ALS patients [43–49]. Intriguingly, many chemokines and their receptors are increased during regenerative myogenesis *in vivo*, and some have been shown to stimulate myoblast migratory activity [50, 51]. However, chemokine expression in ALS muscle remains unexplored.

Clinical and pathological similarities between sporadic and inherited ALS have created the anticipation that models of FALS may provide insight into mechanisms of both forms of the disease and, ultimately, contribute to therapeutic strategies in humans. Skeletal muscle is regarded as one of the most promising targets for therapeutic interventions including molecular therapy, although considerable obstacles remain to be overcome as well [23]. To elucidate potential alterations to myogenic factors in mSOD1-expressing muscle, we systematically investigated transcript and protein level changes in *Pax7* and MRF at different ages of SOD1-G93A mice. Additionally, we looked for transcriptional alterations in muscle chemokine signaling, which may provide alternative cues for myogenic precursor recruitment in ALS.

Materials and Methods

All experimental procedures were approved by the ethics committee of the institution and followed the international guidelines for the use of laboratory animals. The transgenic mice B6SJL-Tg(SOD1-G93A)1Gur/J [4] expressing a high copy number of the

G93A mutant form of human SOD1 were purchased from The Jackson Laboratory (Bar Harbor, Me., USA) and were housed under a 12-hour light/12-hour dark cycle at 21–23°C with a relative humidity of 55%. Food and water were available ad libitum. The transgenic colony was maintained by breeding hemizygous SOD1-G93A males with B6SJL wild-type female littermates. The genotypes were determined from tail samples as described in the Jackson Laboratory protocol. Heterozygous male mice and their nontransgenic male littermates were used for all experiments.

Surgical Denervation

Six male wild-type (nontransgenic) mice of the B6CLJ strain were anesthetized (pentobarbital 30 mg·kg⁻¹, i.p.) at the age of 60 days, and muscle denervation was performed unilaterally in the right hindlimb by extracting a 5-mm segment of the sciatic nerve through an incision in the mid-posterolateral area of the thigh. The left limb was left intact and served as a control. The incision was closed with silk sutures and washed daily with antibacterial solution to prevent infection. The absence of the toe-spreading reflex was confirmed daily. Two weeks after surgical denervation (at the age of 74 days), the animals were sacrificed by cervical dislocation and the gastrocnemius muscles were dissected and frozen immediately in liquid nitrogen.

Extraction of Muscle Samples

Hemizygous SOD1-G93A males and age-matched nontransgenic wild-type control males were sacrificed by cervical dislocation at postnatal (P) age of P40, P60, P90 or P120 days. At each age, 5 animals per genotype were used. Quadriceps, gastrocnemius and soleus muscles from both hindlimbs were individually dissected, snap frozen in liquid nitrogen, and then temporarily stored at -70°C. The quadriceps muscle group (vastus lateralis, vastus medialis and vastus intermedius) and gastrocnemius muscles (medial and lateral) both consist of approximately 60% fast-fatigable (FF) fibers, 35% fast fatigue-resistant fibers and 5% slow (S) fibers, whereas the soleus is composed of 58% S fibers and 42% fast fatigue-resistant fibers [52]. This group of muscles was selected to ensure enough material for both RNA and protein studies, and because it consists of muscles with a high percentage of FF fibers or S fibers, which are most vulnerable or resistant to denervation in the SOD1-G93A model, respectively [8–10]. The dissected muscles from each mouse were pooled and pulverized in a cold mortar with liquid nitrogen. The powdered tissue was divided equally in two prechilled tubes, one for RNA extraction and the other one for protein extraction.

Quantification of mRNA Expression

For total RNA extraction, powdered muscle tissue was further homogenized using a PRO200 homogenizer (PRO Scientific Inc.) and then processed according to the TRIzol Reagent protocol (Invitrogen). The RNA samples were processed with the Turbo DNA-free kit (Ambion) to eliminate genomic DNA. Reverse transcription was carried out according to the SuperScript™ First-Strand Synthesis System kit (Invitrogen) using random hexamers with 1 µg of template RNA in a final volume of 20 µl. TaqMan® (Applied Biosystems) probe-based quantitative PCR reactions were performed from 1 µl of 1:10 diluted cDNA in a reaction mixture containing 1× TaqMan Universal PCR Master Mix (No AmpErase® UNG) and 1× TaqMan primer/probe mix in a final volume of 10 µl. Triplicate reactions from each cDNA were per-

formed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following thermal cycling parameters: incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reaction efficiencies of the primer/probe sets approached 100% (see online supplementary table 1, www.karger.com/doi/10.1159/000324159). In the same conditions, reference gene amplification was performed using TaqMan primer/probe mixtures for three reference genes (18S rRNA, Gapdh and β-actin). Relative gene expression was determined using the 2^{-ΔΔCt} method, where the data are presented as a fold change in gene expression normalized to the reference genes and relative to the nontransgenic age-matched calibrator [53] (online suppl. materials and methods). Analysis of *Ccl5* (Mm01302428_m1), *Ccl2* (Mm00441242_m1) and *Cxcr4* (Mm01292123_m1) transcript levels was carried out in identical reaction conditions but using Applied Biosystems Custom TaqMan Microfluidic Cards and 7900HT Fast Real-Time PCR Systems. Data processing and analysis were carried out as detailed above for the individual reaction assays.

Western Blot Analysis

The powdered muscle tissue was suspended in ice-cold buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml leupeptin/aprotinin and 1 µg/ml pepstatin. The samples were centrifuged at 3,000 rpm at 4°C for 10 min, and the total protein in the supernatant fractions was quantified by the BCA method (Sigma-Aldrich). The proteins (60 µg per well) were separated by SDS-PAGE alongside molecular weight standards, and subsequently transferred to polyvinylidene fluoride membranes (GE Healthcare) at 100 V for 1 h in a transfer buffer containing 25 mM Tris, 200 mM glycine and 10% (v/v) methanol. The membranes were blocked in Tris-buffered saline supplemented with 0.1% Tween 20 and 5% (w/v) powdered skim milk for 1 h at room temperature and incubated overnight at 4°C with a primary antibody diluted in a freshly prepared blocking buffer. The antibody dilutions used were: PAX7 (Ab34360; Abcam) 1:250; MYOD1 (sc-304; Santa Cruz Biotechnology Inc.) 1:300; MYOG (sc-576; Santa Cruz Biotechnology) 1 µg/ml; MYF5 (sc-302; Santa Cruz Biotechnology) 1:200, and GAPDH (sc-25778; Santa Cruz Biotechnology) 1:5,000. Subsequently, the membranes were incubated for 1 h at room temperature in a solution containing the horseradish peroxidase-conjugated secondary antibody (sc-2004; Santa Cruz Biotechnology), diluted 1:5,000 in blocking buffer. Several washes with Tris-buffered saline and 0.1% Tween 20 were performed after incubation with primary and secondary antibodies. Blots were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and were exposed to Curix RP2 Plus X-ray film (Agfa). The computer-assisted intensity analysis of the detected bands was performed with the AlphaEaseFC software (Bonsai Technologies Group). The mean value of normalized intensities from SOD1-G93A animals was compared to that of the wild-type mice to obtain a fold change. For standard errors of the mean, each normalized SOD1-G93A value was compared to the normalized mean intensity of the wild type.

Statistics

Statistics were performed using Statistica 5.0 (Statsoft). Comparisons were carried out by the Student t test, and p < 0.05 was considered significant.

Fig. 1. Upregulation of *Pax7*, MRF, *Rrad* and *Chrna1* transcripts in SOD1-G93A and denervated muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (WT; white bars) at each indicated age (P40, P60,

P90 and P120), or relative to the nonoperated limb in denervated mice (DEN; black bars). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$. **a** *Pax7*. **b** *Myf5*. **c** *Myod1*. **d** *Myog*. **e** *Rrad*. **f** *Chrna1*.

Results

Transcriptional Upregulation of *Pax7* and MRF in *mSOD1* Muscles

To gain insight into the regulation of the myogenic process in SOD1-G93A mice at different stages of the disease, quantitative RT-PCR was performed at P40, P60, P90 and P120. These time points were selected to monitor changes at early (P40) and late presymptomatic (P60) stages, as well as at the onset of the symptomatic (P90) and terminal stages (P120). Relative expression (fold difference) of *Pax7*, *Myf5*, *Myod1* and *Myog* (fig. 1a–d) was determined for each age, using RNA derived from pooled quadriceps, gastrocnemius and soleus muscles of SOD1-G93A hemizygotes and their age-matched nontransgenic littermates. In *mSOD1*-expressing muscle, the production of reactive oxygen species (ROS) is elevated, which in turn activates the expression of *Rrad* (Ras-related associated with diabetes), an early marker in ALS patients

and *mSOD1* mice [37]. Although *Rrad* is also upregulated by denervation, its transcription precedes that of the ‘ROS-insensitive’ denervation marker *Chrna1* (cholinergic receptor, nicotinic, alpha) [37]. Monitoring of *Rrad* (fig. 1e) enabled us also to estimate the first appearance of the muscle damage before the onset of symptoms. Similarly, the extent of denervation was monitored by induction of *Chrna1* (fig. 1f) as this receptor is transcriptionally regulated by electrical activity [32, 34, 54] and not by ROS [37]. To compare the effects of surgical axotomy with those caused by SOD1-G93A mutation, we also performed sciatic denervation on wild-type mice at P60 and analyzed the expression of the above-mentioned factors in the gastrocnemius muscles at P74, 2 weeks after the operation (fig. 1a–f, black bars).

At the early presymptomatic stage of the SOD1-G93A mice (P40), none of the genes under study were significantly affected (fig. 1a–f). Unaltered expression of *Rrad* and *Chrna1* at this time point indicates that muscle dam-

Fig. 2. Protein level regulation of PAX7 and MRF in SOD1-G93A and denervated muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (white bars) at each indicated age (P40, P60, P90 and P120), or

relative to the nonoperated limb in denervated mice (DEN; black bars). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$. Representative Western blot membranes are shown in online supplementary figure 1. **a** PAX7. **b** MYF5. **c** MYOD1. **d** MYOG.

age (and associated ROS production) and denervation in SOD1-G93A animals either has not commenced or is minimal, which is consistent with previously reported data [10, 37]. At the later presymptomatic stage (P60), *Pax7* transcript levels were 3-fold higher than in the non-transgenic controls (fig. 1a). However, *Myf5* (fig. 1b) or *Myod1* (fig. 1c) were not affected, whereas *Myog* (fig. 1d) and *Rrad* (fig. 1e) – but not *Chrna1* (fig. 1f) – were modestly upregulated. At the onset of ALS-like symptoms in the SOD1-G93A animals (P90), a 3-fold upregulation of *Myf5* (fig. 1b), a 2.1-fold upregulation of *Myod1* (fig. 1c) and a robust 10.6-fold upregulation of *Myog* (fig. 1d) was observed, whereas *Pax7* mRNA (fig. 1a) was not significantly affected at this stage. Also, a 11.6-fold induction of *Chrna1* (fig. 1f) was first seen at this age, where the loss of NMJ is already significant. At the onset of the terminal stage (P120), all genes studied were upregulated, ranging from 1.8-fold for *Pax7*, 2.3-fold for *Myf5*, 6-fold for *Myod1* to 20-fold for *Myog*. All genes studied were also upregulated in the denervated gastrocnemius muscles of the nontransgenic wild-type mice compared with the intact control muscles from the same animals (fig. 1a–f, black bars). These observations provide the first evidence of a systematic transcriptional activation of the myogenic program in SOD1-G93A animals and suggest a great degree of similarity in the regulation of MRF mRNA in denervated and mSOD1 muscles. However, as observed earlier in mouse models of ALS [37], *Rrad* was upregulated

earlier than *Chrna1*, suggesting that mSOD1-mediated oxidative stress in the muscle temporally precedes that caused by denervation.

Blunted Increment of Pax7 and MRF Proteins in mSOD1 Muscles

Induction of myogenic factor mRNA in SOD1-G93A mice prompted us to investigate whether the increase in *Pax7* and MRF transcripts was also reflected in the protein level. Western blot analysis was carried out on the same powdered muscle samples as for the mRNA analysis to eliminate possible individual variation between the animals (see Materials and Methods section). At the early presymptomatic stage (P40), the proteins studied were not significantly altered (fig. 2a–d). After this stage, a rather different picture emerged from the protein level studies of PAX7 and MRF as compared with the transcript data. Despite a significant upregulation of *Pax7* (and to a lesser extent *Myog*) mRNA at P60, the corresponding protein levels were not increased (fig. 2a, d). In contrast, MYF5 was 3.5-fold upregulated (fig. 2b) despite unaltered *Myf5* mRNA levels at this stage. At P90, PAX7 (fig. 2a), MYF5 (fig. 2b) and MYOD1 (fig. 2c) proteins were modestly but significantly upregulated, whereas the increment in MYOG was suggestive but not significant (fig. 2d). Most surprisingly, the uniform increase in MRF mRNA levels at P120 was not reflected at the protein level despite the 2.5-fold, 6-fold and 20-fold increases in

Fig. 3. Altered levels of *Ccl5* (a), *Ccl2* (b) and *Cxcr4* (c) transcripts in SOD1-G93A muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (WT; white bars) at each indicated age (P40, P60, P90 and P120). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$.

Myf5, *Myod1* and *Myog* mRNA, respectively. Although MYOG protein showed a tendency to increased relative abundance at advanced stages of the disease, the changes were not significant for any age. In denervated mice (fig. 2a–d, black bars), PAX7, MYF5 and MYOD1 proteins were not significantly altered despite increased mRNA levels. However, MYOG, which was transcriptionally up-regulated in denervated muscle, also showed a 2-fold increase in protein level upon denervation.

These observations reveal both similarities and differences between denervated and SOD1-G93A muscles. PAX7, MYF5 and MYOD1 protein levels were increased at P90 in SOD1-G93A muscles, whereas this was not observed in nontransgenic mice that had been denervated for 2 weeks. The opposite was true for MYOG protein, which was not significantly increased in the mSOD1 muscles at any point but was clearly upregulated upon denervation. Therefore, although transcriptional changes in denervated and mSOD1 muscles are largely consistent (all upregulated), the MYOG protein induction (or lack of it) suggests that differences may exist between SOD1-G93A and denervated muscles.

Altered Expression of Ccl5, Ccl2 and Cxcr4 in SOD1-G93A Muscle

It is intriguing that *Pax7* mRNA was increased in SOD1-G93A muscles already at 60 days of age (fig. 1a). This increase was not likely to be denervation induced because MRF or *Chrna1* mRNA, which respond transcriptionally to surgical denervation (fig. 1, black bars), were not altered at this stage. This led us to investigate an alternative explanation for the origin of the early increment in *Pax7* mRNA expression. Because the *Ccl5* chemokine is upregulated in ALS patients [47] and has the potential to stimulate myoblast migratory activity [50], it was regarded as a promising candidate for the recruitment of cells with myogenic potential by the injured mSOD1 muscle. Compared to the age-matched wild-type muscle, *Ccl5* mRNA was 29-fold elevated in SOD1-G93A muscles at P60 (fig. 3a), which coincided with a 3-fold increase in *Pax7* transcripts (fig. 1a). At this stage, the expression of *Rrad* (fig. 1e) was 8-fold higher in SOD1-G93A animals, revealing the commencing muscle damage. These results support the hypothesis that presymptomatic mSOD1-mediated toxicity may cause the recruitment of circulating myogenic cells to the muscle, which may then partly contribute to the *Pax7*-expressing muscle progenitor pool. To further investigate chemokine-mediated signaling in muscle tissue of SOD1-G93A animals, we looked for mRNA expression of another chemokine, *Ccl2*, which is known to be upregulated in ALS [48, 49] and during in vitro myogenesis [51]. Additionally, we examined the mRNA expression of *Cxcr4*, a chemokine receptor that – together with its ligand, stromal cell-derived factor 1 (SDF-1) – is known to mediate the migration and fusion of muscle cells in vitro and may regulate adult regenerative myogenesis [51]. Unlike *Ccl5*, levels of which were relatively higher in SOD1-G93A animals only at presymptomatic

stage P60, both *Ccl2* (fig. 3b) and *Cxcr4* (fig. 3c) were increased at late stages, starting from the symptomatic stage. These results suggest that, as observed in other tissues of ALS patients, the chemokines *Ccl5* and *Ccl2* are affected in the mSOD1 muscle, although their increased expression is observed at different stages of the disease. Additionally, the results implicate the *Cxcr4*-SDF-1 axis as a potential contributor to SOD1-G93A disease progression.

Discussion

A direct influence of skeletal muscle tissue on pathogenesis in the mouse models of ALS has recently been demonstrated [21, 22]. Here, we investigated the expression of primary determinants of the regenerative potential of the muscle, i.e. SC function (*Pax7*), myoblast proliferation (*Myf5*, *Myod1*) and differentiation (*Myog*) in the SOD1-G93A model of ALS. A gross increment in MRF species was evident at the symptomatic stage (P90), later than the upregulation of *Rrad* and in parallel with the induction of denervation marker *Chrna1*. The drastic increase in MRF transcripts in terminal (P120) SOD1-G93A animals did not result in increased levels of MRF proteins, suggesting that the attempted regeneration of SOD1-G93A muscle by MRF transcript upregulation is futile. Presymptomatic increase in *Pax7* mRNA coincided with the upregulation of chemokine *Ccl5*. Furthermore, we demonstrated an increased mRNA expression of chemokine *Ccl2* and chemokine receptor *Cxcr4* in SOD1-G93A muscle at later disease stages, which mirrored those of the MRF transcripts. In the following discussion, we will first highlight the similarities and differences between SOD1-G93A and denervated muscle. Then, we will discuss the levels of molecular regulation and posttranscriptional mechanisms that may underline the discrepancies in MRF transcript and protein levels and potentially exacerbate the ALS-associated muscle atrophy. Finally, we will discuss how the increased chemokine expression in mSOD1 muscle may contribute to the availability of myogenic precursors in the mutant muscle.

MRF transcripts, and occasionally proteins, have been shown to be upregulated in response to denervation [32–36, 55–58]. Here, the upregulation of MRF transcripts in muscles from denervated limb and their increased relative abundance in SOD1-G93A muscles towards the end of the disease paralleled that of *Chrna1*, suggesting that increments in *Myf5*, *Myod1* and *Myog* transcripts are

mainly due to decreased electrical stimulation caused by denervation. Based on proportional muscle mass in the pooled gastrocnemius, quadriceps and soleus muscles, the contribution of FF fibers was expected to be close to 60%, although this may have shifted towards more fatigue-resistant types in older SOD1-G93A mice [59]. Therefore, rapid transcriptional induction of MRF probably indicates changes in FF fibers that are innervated by the most vulnerable motor axons in mSOD1 mice [9, 10, 60]. The MRF studied (but not SC-specific *Pax7*) are also known to be expressed both in activated myoblasts and in mature myofibers upon denervation [55, 57, 61]. How the described increase in MRF in mSOD1 muscles reflects transcription in SC versus myofibers remains currently unknown.

After 2-week denervation, the MYOG protein level was upregulated in wild-type mice, whereas PAX7, MYF5 and MYOD1 were unaffected. A postdenervation period of 2 weeks was tested here to evaluate the effects of moderately long-term denervation, which we reasoned would better compare with the situation in SOD1-G93A animals. Because MRF expression is strongly induced within days after sciatic denervation and decreases thereafter [32, 34, 35], it is possible that the time point used here was too late to detect increased MYF5 and MYOD1 proteins. Discrepancies between MRF transcript and protein levels have also been reported earlier [62, 63]. Because of a general lack of simultaneous RNA/protein level studies and a wide variety of techniques and experimental species used, a universally valid MRF response to denervation cannot be stated [64]. However, the fact that *Myog* transcripts were equally upregulated in denervated and SOD1-G93A muscle, but the MYOG protein increase was only apparent upon denervation, suggests that posttranscriptional regulation of this factor in SOD1-G93A mice may be altered.

Several molecular and pathophysiological alterations may contribute to the discordance between MRF mRNA and protein levels in SOD1-G93A mice. Although MRF proteins were not upregulated at the terminal stage, they were not depleted either (fig. 2). MRF can increase their own transcription and cross-regulate one another's expression [65]. The transcription factor activity of MYOD and MYOG is regulated by phosphorylation [66, 67], relies on hetero-oligomerization with E-proteins [68], and is inhibited by Id1, a negative regulator of MRF/E-protein oligomerization [69]. Therefore, elevated transcription of MRF mRNA in SOD1-G93A muscles could potentially result from the altered phosphorylation status of the MRF proteins or, alternatively, from the changed balance be-

tween their oligomerization partners and inhibitors, such as E-proteins and Id1.

Skeletal muscle atrophy induced by pathological conditions involving oxidative damage is characterized by increased proteolysis via the ubiquitin-proteasome pathway [70, 71]. Consistently, the expression of ubiquitin ligases is elevated in human ALS patients and mSOD1 mice [72]. Both MYOD1 and MYOG are targets for proteasomal activity [73, 74], and ubiquitin-proteasome-mediated degradation of MYOD1 occurs in several models of skeletal muscle atrophy [75]. Interestingly, ubiquitin ligases in denervated muscle are under direct transcriptional control of MYOG, which therefore may promote the loss of muscle mass as well as differentiation [76]. Muscle-specific expression of insulin-like growth factor 1 attenuates muscle wasting and reduces protein ubiquitination [77], which is associated with increased PAX7 and MYOG protein expression at the terminal stage of SOD1-G93A mice [78]. Therefore, insulin-like growth factor 1 may alleviate the metabolic processes that lead to the blunted protein level expression of these factors in SOD1-G93A muscle. Genetic or pharmaceutical targeting of the proteasome or associated enzymes could possibly serve as a candidate strategy to alleviate SOD1-G93A muscle pathology.

Rather than indicating direct transcriptional activation, increased MRF transcript abundance also derives from posttranscriptional mRNA stabilization. Both *Myod1* and *Myog* transcripts are targets of the mRNA-stabilizing enzyme HuR, which is elevated in regenerating myofibers in vivo [79]. Under endoplasmic reticulum (ER) stress, global changes in transcript abundance are largely controlled by mRNA stability. Although a possible contribution of ER stress to the pathobiology of the ALS muscle remains unexplored, it has been shown to take place in affected neurons of human ALS patients and mSOD1 models [80]. Equally, RNA oxidation occurs in neurons of both ALS patients and mice overexpressing mSOD1 [81]. Because mRNA stabilization under ER stress [82] and mRNA oxidation in general [83] are associated with decreased protein translation, they may have a role in the observed disparity between the MRF transcripts and their corresponding proteins in SOD1-G93A muscle.

Besides the regulation of MRF at the late disease stages, a discordance was observed between *Pax7* and *Myf5* transcript and protein levels at the presymptomatic stage. *Pax7* mRNA but not protein was increased at P60, whereas *Myf5* was only increased in protein level (fig. 1, 2). This inconsistency does not derive from muscle sample varia-

tion as the same frozen tissue was used for both RNA and protein experiments (see Materials and Methods section). Although further experiments are required to formally address these observations, they may also be linked. *Pax7* has recently been shown to be a target of the muscle-specific microRNA miR-1 and miR-206 [84], which are upregulated in mSOD1 mice [85]. Inhibition of these microRNA increases the PAX7 protein level in vivo and inhibits differentiation [84]. MYF5 potentially upregulates these microRNA in developmental myogenesis [86] and may therefore inhibit *Pax7* mRNA translation. On the other hand, experiments on synchronized myoblasts have shown that MYF5 protein abundance is regulated by the cell cycle and that the variations in protein level are largely independent of those of *Myf5* mRNA, indicating posttranslational control [87, 88]. This property of *Myf5* regulation may contribute to the increased MYF5 protein level despite a lack of transcriptional increase at P60. More detailed analysis is warranted to test these hypotheses on mSOD1 muscle in vivo.

Within healthy skeletal muscle, the quiescent SC number largely remains constant. The relative increase in presymptomatic *Pax7* expression in SOD1-G93A muscle could indicate denervation-independent SC renewal/activation or, alternatively, recruitment of *Pax7*-expressing cells by muscle from other stem cell niches. Myogenic precursor recruitment could potentially be enhanced by chemokines such as *Ccl5*, which is upregulated in ALS patients [47]. *Ccl5* can stimulate myoblast migratory activity [50], and its transcripts were markedly increased at the presymptomatic stage in SOD1-G93A mice (fig. 3a). Increases at the symptomatic and terminal stages of the disease in chemokine *Ccl2* (fig. 3b), also increased in ALS patients [48, 49], and in chemokine receptor *Cxcr4* (fig. 3c) provide further support for altered chemokine-mediated signaling in SOD1-G93A muscles. *Cxcr4* is expressed in numerous adult stem cells [89], and its ligand, SDF-1, is known to be released from regenerating muscle to attract CXCR4-positive cells [90]. Although these findings are only correlative at this point, they encourage further experiments investigating the nature and regulation of potential myogenic precursor recruitment by chemokines in SOD1-G93A.

To conclude, we described the induction of the myogenic program in the course of the disease in SOD1-G93A mice and revealed both denervation-dependent and -independent effects on *Pax7* and MRF expression. These findings encourage further studies on the myogenic process in ALS and indicate that some caution may be required when data are interpreted solely based on tran-

script abundance. The blunted protein level accumulation of MRF described here should also guide future research in the evaluation of posttranscriptional and posttranslational mechanisms regulating myogenesis in mSOD1 muscles. Furthermore, research aiming at a detailed understanding of chemokine-mediated signaling in affected ALS muscles may help to improve the delivery of myogenic precursors to muscle tissue, which is of considerable interest to those working on therapeutic aspects of ALS.

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