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Early vulnerability to ischemia/reperfusion injury in motor terminals innervating fast muscles of SOD1-G93A mice

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Abstract

In mouse models of familial amyotrophic lateral sclerosis (fALS) motor neurons are especially vulnerable to oxidative stresses *in vitro*. To determine whether this increased vulnerability also extends to motor nerve terminals *in vivo*, we assayed the effect of tourniquet-induced ischemia/reperfusion (I/R) injury on motor terminals innervating fast and slow hindlimb muscles in male G93A-SOD1 mice and their wild-type littermates. These mice also expressed yellow fluorescent protein (YFP) in motor neurons. We report that in SOD1-G93A/YFP mice the motor terminals innervating two predominantly fast muscles, extensor digitorum longus (EDL) and plantaris, were more vulnerable to I/R injury than motor terminals innervating the predominantly slow soleus muscle. The mean duration of EDL ischemia required to produce a 50% reduction in endplate innervation in SOD1-G93A/YFP mice was 26 min, compared to 45 min in YFP-only mice. The post-I/R destruction of EDL terminals in SOD1-G93A mice was rapid (< 2 hr) and was not duplicated by cutting the sciatic nerve at the tourniquet site. The increased sensitivity to I/R injury was evident in EDL muscles of SOD1-G93A/YFP mice as young as 31 days, well before the onset of motor neuron death at ~90 days. This early vulnerability to I/R injury may correlate with the finding (confirmed here) that in fALS mice motor nerve terminals innervating fast hindlimb muscles degenerate before those innervating slow muscles, at ages that precede motor neuron death. Early vulnerability of fast motor terminals to I/R injury thus may signal, and possibly contribute to, early events involved in motor neuron death.

INTRODUCTION

Most studies of mechanisms underlying motor neuron death in the neurodegenerative disease ALS have focussed on changes occurring within the spinal cord. However in animal models of familial ALS (e.g. mice expressing mutant forms of human superoxide dismutase I, SOD1) there is structural and functional evidence for damage to motor axons and motor nerve terminals that precedes the onset of paralysis and motor neuron death (Chiu et al., 1995; Azzouz et al., 1997; Kennel et al., 1996; Kong and Xu, 1998; Frey et al., 2000; Fischer et al., 2004; Sasaki et al., 2004; Wooley et al., 2005; Pun et al., 2006; Boillée et al., 2006; Gould et al., 2006). Fischer et al. (2004) presented evidence for more extensive peripheral than central morphological damage in a patient diagnosed with sporadic ALS who died prematurely. Early distal defects have also been demonstrated in hereditary canine spinal motor atrophy (Rich et al., 2002). This distal damage contributes to motor symptoms and might also accelerate motor neuron death, an hypothesis supported by findings that certain types of mechanical injury to

peripheral nerves can accelerate disease progression in SOD1-G93A mice (Mariotti et al., 2002; Sharp et al., 2005).

Due to their peripheral location, motor nerve terminals may be subjected to stresses not normally experienced by the parent motoneuron, such as transient partial ischemia during intense (anaerobic) muscle activity or I/R injury following temporary interruption of limb blood supply. When hindlimbs of wild-type rodents are subjected to I/R injury, motor nerve terminals are the most vulnerable neuromuscular component. For example, electron microscopic studies of rat limbs examined 1 day – 4 weeks following 2 hr tourniquet ischemia detected no changes in axons, Schwann cells or muscle fibers, but multiple signs of degeneration in motor nerve terminals, including disruption of presynaptic membrane, degeneration of mitochondria and the presence of vacuoles (Tombol et al., 2002). Makitie and Teravainen (1977) detected swelling of motor terminal mitochondria after a similar stress. Functional measurements also show that the neuromuscular junction is a major site of I/R injury (e.g. Eastlack et al., 2004). Mechanisms underlying the special vulnerability of motor terminals to I/R stress have not yet been determined.

The present study tested whether motor terminals in the G93A-SOD1 mouse model of familial ALS are more vulnerable to I/R injury than wild-type motor terminals, and if so, when this extra vulnerability first becomes evident. We also tested whether motor terminals that innervate different muscle types are differentially sensitive to I/R injury, since Frey et al. (2000) and Pun et al. (2006) demonstrated in SOD1-G93A mice that the hindlimb motor terminals that innervate fast (types IIa and IIb) muscle fibers degenerate earlier than those innervating slow, fatigue-resistant fibers (type I). We show that in SOD1-G93A mice the motor terminals innervating two predominantly-fast hindlimb muscles are more vulnerable to tourniquet-induced I/R injury than those innervating a slow hindlimb muscle. This increased vulnerability becomes evident more than a month before the onset of paralysis or motor neuron death.

MATERIALS AND METHODS

Mice

SOD1-G93A/YFP mice were generated by crossing two transgenic strains obtained from Jackson Labs (Bar Harbor, ME): B6.Cg-TgN(SOD1-G93A)1Gur/J (stock #4435), which express a high copy number of this mutant human SOD1 in all cells, and B6.Cg-Tg(Thy1-YFP)16Jrs/J (stock number #3709), which express YFP in many neurons, including spinal motor neurons, but not in muscle or Schwann cells. YFP expression facilitates assessment of motor terminal innervation for large populations of endplates and does not reduce the viability of SOD1-G93A mice, as also reported by Schaefer et al. (2005). Tail clips obtained at postnatal days 18–20 (P18–20) were used to identify SOD1-G93A mice by RT-PCR (as described in Vila et al., 2003), and to identify YFP expression (in sensory axons) by fluorescence microscopy. A genotype identification code was tattooed on each mouse (AIMS Tattoo System, Hornell, NY). Since most SOD1-G93A mice were studied before signs of weakness/paralysis became apparent, PCR was repeated at the time of the experiment to confirm genotype. We used male mice (ages P24–121) which expressed both SOD1-G93A and YFP and their YFP-only littermates. Only male mice were used to ensure more uniform results, since disease progression is reported to be slower in female than in male SOD1-G93A mice (Veldink et al., 2003; Miana-Mena et al., 2005), and in wild-type mice females are more resistant to CNS I/R injury than males (Sampei et al., 2000; Bramlett, 2005).

Ischemia/reperfusion (I/R) protocol

Mice were anesthetized by subcutaneous injection of 5 µl/g of a solution containing ketamine (5 mg/ml) and xylazine (0.5 mg/ml), and placed on a heating pad maintained at 38 °C (Harvard

Apparatus, Holliston, MA). Tourniquet ischemia was applied to one hindlimb by tying an elastic rubber band (3 mm width) around the thigh at the level of the greater trochanter. There was no direct pressure on EDL, plantaris or soleus muscles, which are below the femorotibial joint. The tension of the tourniquet was adjusted to be sufficient to interrupt the arterial pulse in the limb, monitored using a homemade optical pulse meter (footpad placed between a light-emitting diode and a photoresistor) that converted the pulse wave into voltage changes on an oscilloscope screen. Interruption of the arterial blood supply was indicated by disappearance of the pulse waves and reduction of the footpad skin temperature by $\sim 2^{\circ}\text{C}$. Reperfusion following tourniquet removal was indicated by reappearance of the pulse waves. The ischemic period lasted 15–60 min; anesthetic was re-injected for ischemic durations >30 min. Mice were sacrificed by inhalation of 100% CO_2 6–24 hr following the onset of reperfusion, except as noted. All procedures were approved by the Animal Care and Use Committee of the University of Miami.

Muscle dissection and staining of endplates

Control and post-I/R hindlimbs were removed by cutting below the ilio-femoral joint. EDL and soleus (and in 2 cases plantaris) muscles were exposed by rapidly (~ 5 min) removing the skin and dissecting away the overlying muscles in normal mouse saline (NMS) containing (in mM): NaCl 137, NaHCO_3 15, KCl 4, CaCl_2 1.8, MgCl_2 1.1, NaH_2PO_4 0.33, and glucose 11.2, equilibrated with a 95% O_2 /5% CO_2 gas mixture, pH 7.4. Limbs were then fixed at room temperature in freshly prepared 4% paraformaldehyde (in phosphate-buffered saline) for 40 min and rinsed in NMS for 30 min. Muscles were removed by cutting their tendons. Endplate acetylcholine (ACh) receptors were labeled with 25 $\mu\text{g}/\text{ml}$ α -bungarotoxin (α -BgTx) Alexa Fluor 594 conjugate (Invitrogen, Carlsbad, CA) for 30 min and rinsed with NMS for 40 min. This is a good marker for endplates because junctional ACh receptors turn over very slowly, even after denervation (Bevan and Steinbach, 1983). Each muscle was separated into 4–5 longitudinal segments and mounted between #1 glass coverslips (Thomas Scientific) with hard mounting medium (Vectashield, Vector Laboratories, Burlingame, CA).

EDL and soleus muscles have different fiber type compositions. In the C57BL/6J strain studied here, soleus contains predominantly type I fibers (slow, oxidative, 34–40%) and type IIa fibers (fast, fatigue-resistant, mixed oxidative and glycolytic, 55–59%, Totsuka et al., 2003; Luedeker et al., 2004). In contrast, EDL contains mainly type IIb (fast, fatiguable, glycolytic, 78%) and intermediate (type IIx, 20%) fibers (Luedeker et al., 2004). Like EDL, plantaris contains mainly types IIb and IIx fibers (83%, Waters et al., 2004). Muscle fiber type is determined mainly by the activity pattern of the innervating motor neuron; type IIb fibers are innervated by FF motor neurons that innervate fast, fatiguable motor units; type IIa fibers are innervated by FR motor neurons that innervate fast, fatigue-resistant motor units; and type I fibers are innervated by S motor neurons that innervate slow, fatigue-resistant motor units (reviewed by Burke, 2004). Thus EDL and plantaris are innervated mainly by FF (and perhaps FR) motor neurons, and soleus is innervated by FR and S motor neurons. Expression of SOD1-G93A may alter these fiber type distributions as the disease progresses (Frey et al., 2000; Derave et al., 2003; Atkin et al., 2005), but is unlikely to have any major effect at the earliest (P30–P40) presymptomatic stages assayed here.

Imaging and calculation of endplate occupancy

Images were obtained with a confocal system that included a Yokogawa spinning disk (Solamere, Salt Lake City, UT), and a Cascade 512B camera (Roper Scientific, Tucson, AZ) mounted on a Nikon TE2000E microscope (Nikon, Melville, NY). Images were obtained using a 60X water immersion lens (NA 1.2, Olympus, Melville, NY) and a 4X lens (NA 0.2, Nikon). YFP and α -BgTx were excited at 488 or 568 nm, respectively (argon/krypton laser, Laser Physics, West Jordan, UT). Emitted light was filtered with a 535 nm band pass filter (40 nm

bandwidth) for YFP or a long-pass 590 nm filter for α -BgTx (both filters from Chroma, Rockingham, VT). Images were acquired using IPLAB (v. 3.6, Scanalytics Inc, Fairfax, VA) using 0.5 s exposures. Muscle fields containing endplates were identified by their α -BgTx labeling, and 30–50 endplates were imaged for each muscle. For each field YFP and α -BgTx image pairs were acquired for 15–25 Z sections separated by 1 μ m in the vertical dimension, using custom-written scripts in IPLAB.

Measurements of endplate and motor terminal areas were made using custom programs written in V-Pascal with V++ imaging software (Digital Optics, Auckland, New Zealand). For each endplate one YFP and one α -BgTx image were generated from the series of Z sections by creating a maximal projection image onto the XY plane. Endplate area was calculated from outlines traced around the α -BgTx image (see Fig. 1A,B). The corresponding motor terminal area was calculated after tracing the outlines of the largest YFP-filled structure contiguous with the preterminal axon. Endplate occupancy was calculated in Excel as the ratio of terminal and endplate areas. Since in YFP-only mice the area of the α -BgTx projection image usually exceeded that of the corresponding YFP projection image, the mean endplate occupancy calculated for a fully innervated, non-stressed muscle was usually less than 1, with a mean of 0.88 (see e.g. Fig. 3).

To estimate the ischemia duration at which endplate occupancy was reduced to 50% of maximal (ischemia duration 50, ID₅₀), plots of mean endplate occupancy as a function of ischemia duration (Fig. 3) were fitted with curves using a sigmoidal equation:

$$O = O_{\min} + (O_{\max} - O_{\min}) / (1 + (ID_{50} / t)^s),$$

where O = mean occupancy, O_{min} is the minimal measured mean occupancy (0), O_{max} is maximal occupancy measured in non-stressed muscles, t is the duration of ischemia in min, and s is slope. The best-fitting value of s (−8.0) was used for all plotted curves.

Statistics

Plots and histograms were created using Prism (version 3, Graph Pad Software, San Diego, CA), which was also used to perform tests of statistical significance. Non-parametric tests (Mann-Whitney, Kruskal-Wallis with Dunn's post-test) were used for non-Gaussian distributions.

RESULTS

Motor terminals in EDL muscle of SOD1-G93A mice are highly sensitive to ischemia-reperfusion (I/R) injury, even at presymptomatic ages

Fig. 1A,B shows micrographs of EDL muscles from young (P31–P33) YFP-only (A) and SOD1-G93A/YFP (B) mice whose right hindlimbs were subjected to tourniquet-induced ischemia for 30 min followed by 6 hr reperfusion in vivo (Ab, Bb). Non-stressed contralateral EDLs served as controls (Aa, Ba). All endplates in the non-stressed YFP-only muscle appear yellow-orange, indicating that all the α -BgTx-labelled endplates (red) were innervated by a YFP-labelled motor terminal (green). All endplates in the non-stressed SOD1-G93A/YFP muscle were also innervated, consistent with previous studies demonstrating no significant motor terminal degeneration in these mice at one month (Chiu et al., 1995; Frey et al., 2000; Pun et al., 2006). Following the I/R stress, most YFP-only endplates remained innervated, but endplates in SOD1-G93A/YFP EDL appeared mostly red, indicating widespread denervation.

The higher-magnification images in Fig. 1 show separate α -BgTx (Ac-d, Bc-d) and YFP (Ae-f, Be-f) images. Dotted lines illustrate how the endplate region was outlined in the α -BgTx

image, and the corresponding motor terminal (if present) was outlined in the YFP image. These outlined areas were used to calculate the percentage of the endplate area that was occupied by the motor terminal (endplate occupancy). Fig. 1C,D shows histograms (a,b) and cumulative histograms (c) of endplate occupancy for each of the experimental conditions illustrated in Fig. 1A,B, compiled from data pooled from EDLs from mice aged P31–P101. Histograms in non-stressed (control) show a single cluster of endplates with occupancies ranging from ~70–100%. Muscles subjected to the 30 min I/R stress showed this cluster plus another cluster with less than 10% occupancy. Very few terminals exhibited intermediate occupancies. Low-occupancy endplates were rare in stressed YFP-only EDLs, but constituted the majority of endplates in the EDLs of SOD1-G93A/YFP mice. Thus a 30 min I/R stress that produced only minor denervation of EDL endplates in a YFP-only mouse produced nearly complete denervation of EDL endplates in SOD1-G93A/YFP mice, even at young ages.

In SOD1-G93A/YFP mice motor terminals innervating fast muscles are more sensitive to I/R injury than motor terminals innervating a slow muscle

In other hindlimb muscles of SOD1-G93A mice (triceps surae, lateral gastrocnemius) evidence has been presented that motor terminals innervating fast type IIb muscle fibers degenerate faster than motor terminals innervating other muscle fiber types (Frey et al., 2000; Pun et al., 2006). Since the major fiber type in EDL is IIb (see Materials and Methods), we hypothesized that increased motor terminal vulnerability to I/R injury would develop sooner in EDL than in soleus, a slow muscle with few (if any) type IIb fibers.

Fig. 2 plots mean endplate occupancy following a 30 min I/R stress as a function of age in non-stressed and stressed EDL (Fig. 2A) and soleus (Fig. 2B) of YFP-only and SOD1-G93A/YFP mice. Consistent with the above-cited studies, in non-stressed SOD1-G93A/YFP the mean endplate occupancy declined slowly with age in EDL, but not in soleus (compare open circles in Fig. 2Ab,Bb). In stressed SOD1-G93A/YFP limbs, mean endplate occupancy fell sharply with age in EDL, but did not fall in soleus (with one exception; compare filled circles in Fig. 2Ab,Bb). The marked increase in vulnerability to I/R injury in SOD1-G93A/YFP EDL was evident in some muscles as early as P31, and was evident in all sampled muscles older than P40. There was a tendency toward less I/R-induced damage in SOD1-G93A/YFP EDL younger than P30, but we did not examine animals younger than P24. In YFP-only mice the mean endplate occupancy of both non-stressed and stressed EDL and soleus remained high (>70%) for all ages examined (P24–P120, Fig. 2Aa,Ba).

Thus over the tested age range, most motor terminals in SOD1-G93A/YFP soleus showed no apparent damage following an I/R stress that denervated most endplates in EDL. The increased vulnerability to I/R damage in EDL motor terminals of SOD1-G93A/YFP mice became evident weeks before the onset of significant endplate denervation in the contralateral non-stressed EDL, suggesting that increased vulnerability to I/R injury may be an early functional sign of impending motor terminal degeneration.

To test whether increased vulnerability to I/R injury is a general feature of SOD1-G93A hindlimb fast muscles or just a peculiar feature of EDL terminals, we performed two additional experiments that included analysis of plantaris, another predominantly fast hindlimb muscle rich in type IIb fibers (SOD1-G93A mice, P50–52, 46–99 endplates sampled in each muscle). In one mouse the 30 min I/R stress denervated 96% of endplates in EDL, 90% in plantaris, and 7% in soleus. In the other mouse this stress denervated 22% of endplates in EDL, 33% in plantaris, and none in soleus. Denervation in non-stressed limbs was 1%. The different magnitudes of endplate denervation measured in these two mice probably reflects the fact that a 30 min I/R stress falls near the steepest and most variable region of the relationship between ischemia duration and endplate occupancy for EDL (see Fig. 3A, described below). But in both mice the magnitude of the I/R-induced damage in plantaris was similar to that in EDL and

more extensive than that in soleus. This result suggests that increased vulnerability to I/R stress is a general property of fast hindlimb muscles in SOD1-G93A mice.

Fig. 3 plots how the duration of ischemia (0–60 min) affected mean endplate occupancy. Results were fitted with a sigmoidal curve that enabled estimation of the ischemia duration expected to reduce mean endplate occupancy to 50% of that measured in non-stressed muscles (ID_{50} , see Materials and Methods). For EDL the ID_{50} fell from 45 min in YFP-only to 26 min in SOD1-G93A/YFP, a 42% decrease (Fig. 3A). For soleus the SOD1-G93A mutation had only a small effect on the ID_{50} (48 min in YFP-only, 44 min in SOD1-G93A/YFP, Fig. 3B). These measurements thus suggest that in SOD1-G93A/YFP mice >P35, motor terminals innervating fast EDL have much less tolerance for I/R stress than those innervating slow soleus. In contrast, the stress tolerance of these fast and slow muscles is similar in YFP-only mice, resembling that measured in SOD1-G93A/YFP soleus.

Post-I/R denervation of SOD1-G93A endplates is not correlated with endplate area, and is not duplicated by axotomy at the tourniquet site

Endplate areas in EDL varied over a wide range, from <100 to >600 μm^2 . To test whether the area of an endplate influenced its tendency to lose innervation following an I/R stress, we plotted endplate occupancy vs. area for all post-I/R SOD1-G93A/YFP endplates. The slope of the regression line fitted to these data was not significantly different from zero, suggesting that endplate area did not influence post I/R denervation.

Fig. 4 shows results of an experiment to test whether the damage to EDL SOD1-G93A/YFP motor terminals illustrated in Figs. 1–3 might have been due to mechanical injury of axons at the tourniquet site rather than to I/R injury. The sciatic nerve innervating one hindlimb was cut at the level of the sciatic notch; the other hindlimb was subjected to the standard tourniquet-induced 30 min ischemia followed by 6 hr reperfusion. The cumulative histogram of endplate occupancy shows that in the axotomized limb EDL retained a high mean occupancy, in contrast to the almost complete denervation of EDL after the I/R stress. Thus the rapid post-I/R denervation of EDL endplates in SOD1-G93A/YFP mice did not result from axonal injury at the tourniquet site.

Post-I/R denervation of EDL SOD1-G93A endplates develops rapidly

Fig. 5 shows results from an experiment to estimate the time course of motor terminal degeneration following the onset of reperfusion. EDLs from SOD1-G93A/YFP and YFP-only mice were subjected to 30 min ischemia *in vivo*, then reperfused, dissected, transferred to oxygenated mouse saline and imaged over time. Thus in this experiment reperfusion began *in vivo*, and re-oxygenation continued *in vitro* during and following dissection. The YFP-only terminal showed no signs of degeneration 35 or 100 min after reperfusion began (compare a and b in Fig. 5A), but over this same time interval the SOD1-G93A/YFP terminal fragmented and lost continuity with the preterminal axon (Fig. 5Ba,b). Filled circles in Fig. 5Bc plot the post-ischemic time course of the decay of total fluorescence in the SOD1-G93A/YFP terminal, demonstrating the greatest loss between ~80–120 min following the onset of reperfusion. The post-I/R terminal degeneration in the *in vivo* experiments of Figs. 1–4 was likely also complete within this interval or even sooner, because post-I/R mean endplate occupancies measured in SOD1-G93A/YFP terminals showed no significant correlation with *in vivo* reperfusion times ranging from 1–24 hr (1 hr, $n = 2$ muscles; 6 hr, $n = 7$; 24 hr, $n = 2$; Pearson correlation test, two-tailed, $p = 0.16$).

EDL motor terminals in SOD1-G93A mice can resprout following I/R injury

Pun et al. (2006) reported that after a proximal peripheral nerve crush in SOD1-G93A mice, axons could reinnervate fast muscle fibers if the crush occurred at P30, but not if the crush

occurred at P38, suggesting that fast motor axons lose their ability to resprout at an early age. Thus we wondered whether endplates in SOD1-G93A/YFP EDL could become reinnervated following an I/R stress sufficient to denervate endplates. Fig. 6 shows results from an experiment in which the EDL in one hindlimb of a P65 SOD1-G93A/YFP mouse underwent a 30 min ischemic stress followed by 10 days of reperfusion. Almost all endplates in the post-I/R EDL were innervated, and at least some of this innervation represented re-innervation of formerly denervated endplates because a 30 min I/R stress denervates many EDL endplates in SOD1-G93A mice (Fig. 3A). Another sign of reinnervation are the numerous nerve sprouts extending beyond the α -BgTx-delimited endplate region in the post-I/R EDL (arrowheads, e.g. Brown and Ironton, 1978; Brown et al. 1980). Thus at least some of the SOD1-G93A motor axons innervating this fast muscle can resprout following I/R-induced degeneration of their motor terminals.

To determine whether post-I/R reinnervation could also occur in EDLs from older SOD1-G93A/YFP mice, we administered the standard 30 min I/R stress to one hindlimb in each of two P89 littermates, and measured endplate innervation in 10 days later at P99. The fraction of fully occupied EDL endplates in the stressed limb was $47 \pm 5.5\%$ (SEM), significantly less than that in the non-stressed limb ($93 \pm 1.4\%$, $p < 0.001$, Dunn's multiple comparison test applied to measurements from endplates in 15–20 microscope fields, each containing 5–20 endplates). Sprouts were present in $14 \pm 1.9\%$ of the post-I/R endplates, compared to $5.3 \pm 1.8\%$ in the non-stressed limb ($p < 0.001$). These data suggest that even at this relatively late age, at least some EDL axons in SOD1-G93A mice remained capable of reinnervating denervated endplates. However, this reinnervation was incomplete compared to the near complete reinnervation measured in the younger mouse of Fig. 6.

DISCUSSION

In SOD1-G93A mice the motor terminals innervating fast hindlimb muscles become especially vulnerable to ischemia/reperfusion (I/R) injury at an early age

Our results demonstrate that in male mice expressing the G93A mutation of SOD1 the motor terminals innervating two predominantly fast hindlimb muscles, EDL and plantaris, were more vulnerable to I/R injury than terminals in the same muscles of wild-type mice. In contrast, the vulnerability of SOD1-G93A motor terminals innervating soleus muscle was similar to wild-type. The differential vulnerability of motor terminals innervating fast and slow muscles was associated with the SOD1-G93A mutation, because in wild-type mice the sensitivity to I/R injury was similar for EDL and soleus motor terminals. This increased vulnerability of EDL motor terminals to I/R injury in male SOD1-G93A mice began as early as P31, well before the onset of motor neuron death.

The increased vulnerability to I/R injury developed before the onset of degeneration of motor terminals innervating non-stressed EDL muscles (Fig. 2Ab), and thus might reflect a stress-induced acceleration of the disease-related degeneration of motor terminals in fast muscles. This motor terminal degeneration is consistent with the early, selective decline in the number of the most forceful (type IIb) motor units in fast-twitch hindlimb muscles reported in SOD1-G93A mice (Hegedus et al. 2006). Differential denervation of endplates may account for Derave et al.'s (2003) finding of atrophy in EDL but not soleus of SOD1-G93A mice.

The literature suggests several possible reasons why the G93A mutation of SOD1 might make motor terminals innervating fast muscles more vulnerable than those innervating slow muscles. Fast-fatiguable (FF) motor neurons might be more vulnerable because they innervate more muscle fibers than other motor neuron types (Fahim et al., 1984). The fast muscle fibers specified by FF innervation have lower basal blood flows (review by Burke, 2004) and a lower basal expression of vascular endothelial growth factor (VEGF, an angiogenic and motor neuron

trophic factor, Annex et al., 1998; Lambrechts et al., 2003; Storkebaum et al., 2005). Fast muscles release more H₂O₂ than slow muscles (Capel et al., 2004), and SOD1-G93A expression increases production of toxic hydroxyl radicals from H₂O₂ (Yim et al., 1996, 1999). Fast muscles also exhibit higher concentrations of neuronal nitric oxide synthase than slow muscles (Grozdanovic, 2001). Expression of SOD1-G93A sensitizes cultured spinal motor neurons to nitric oxide-induced death (Raoul et al., 2002, 2006).

Could stress-induced damage to motor nerve terminals contribute to motor neuron death in SOD1-G93A mice?

The I/R stress applied here did not kill all EDL motor neurons in SOD1-G93A mice, because EDL endplates were reinnervated within 10 days after a stress administered at P65 (Fig. 6). But repeated bouts of I/R stress might permanently damage motor neurons, since evidence from developmental and reinnervation studies in wild-type rodents indicates that motor neurons become especially sensitive to stress-induced damage during axonal regeneration (reviewed in Sharp et al., 2005). These authors found that crushing a hindlimb nerve, an injury also followed by regeneration of motor terminals, sped the time course of motor neuron death in SOD1-G93A mice. Even if motor neurons remain alive, a peripheral I/R stress could hasten functional disease progression if motor axons fail to reinnervate previously denervated endplates. We found reduced reinnervation of EDL terminals in SOD1-G93A mice stressed at an older age (P89).

If I/R injury to motor terminals can contribute to motor neuron death and/or persisting functional denervation in fALS mice, then one might predict that a history of intense, anaerobic exercise might correlate with more rapid disease progression. Consistent with this idea, in SOD1-G93A mice intense exercise is reported to accelerate disease progression (Mahoney et al. 2004), though moderate exercise tends to slow disease progression (e.g. Kirkinezos et al. 2003; results in males and females sometimes differ). Another circumstance that might predispose to peripheral I/R stress is inability to increase blood flow to exercising muscle. Consistent with this idea, there is selective motor neuron degeneration in mice lacking the hypoxia response element in the VEGF gene (Oosthuysen et al. 2001), and crossing these mice with SOD1-G93A speeds disease progression (Lambrechts et al. 2003). Murakami et al. (2003) found that SOD1-G93A expression impairs hypoxia-induced VEGF expression in presymptomatic mice. Reperfusion following ischemia imposes an oxidative stress, and muscles in SOD1-G93A mice exhibit upregulation of antioxidant enzymes, which might reflect compensation for oxidative stress (Mahoney et al., 2006).

If motor terminals are a significant site of motor neuron damage in fALS mice, then disease onset and/or progression might be slowed by protecting motor terminals. The protective effects of neurotrophic factors expressed by viral constructs injected into SOD1-G93A skeletal muscles have traditionally been attributed to retrograde transport of the trophic factor to motor neuron somata rather than to locally-mediated effects on motor terminals (e.g. Kaspar et al. 2003), but local effects on motor terminals might contribute to the findings that glial-derived neurotrophic factor is more protective when expressed in muscle than when expressed in astrocytes (Li et al. 2006), and that muscular expression of non-retrogradely transported VEGF is also neuroprotective (Lafuste et al. (2006). Also consistent with the idea that peripheral events contribute to disease onset/progression in fALS mice is the finding that protection of motor neuron somata (via deletion of the apoptotic protein BAX) is not sufficient to prevent death of SOD1-G93A mice (Gould et al. 2006).

In sum, our demonstration that motor terminals innervating fast hindlimb muscles in male SOD1-G93A mice become especially susceptible to I/R injury at a young age, combined with other findings cited above, suggests that damage to motor terminals deserves serious consideration as a source of motor neuron damage in fALS mice and possibly in human ALS.

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Fig. 1.

An ischemia/reperfusion (I/R) stress denervates most endplates in the EDL of SOD1-G93A/YFP (B,D), but not YFP-only mice (A,C). One hindlimb in each of 2 male mice (P33 YFP-only; P31 SOD1-G93A/YFP) was subjected to 30 min of tourniquet-induced ischemia followed by 6 hr of reperfusion in vivo. The non-stressed contralateral hindlimb served as a control. The upper, low-power micrographs (Aa-b, Ba-b) are digital overlays of the fluorescence of YFP (green, delineating motor axons and terminals) and Alexa 594-labelled α -BgTx (red, marking endplate ACh receptors) in stressed and non-stressed EDLs. Innervated endplates are yellow-orange; denervated endplates are red. Middle and lower rows show at higher magnification the separate α -BgTx (Ac-d, Bc-d) and YFP (Ae-f, Be-f) images corresponding to the regions indicated by the dotted boxes in Aa-b and Ba-b. Endplates marked by α -BgTx staining were outlined (dotted lines) and the motor terminal innervating each

endplate was outlined in the YFP image, to determine endplate occupancy (see Materials and Methods). Histograms of endplate occupancy in Ca,b and Da,b show results pooled from EDLs of all analyzed YFP-only and SOD1-G93A/YFP mice (P31–P101) subjected to 30 min ischemia followed by 6–24 hr reperfusion. Each histogram is based on measurements from 152–291 terminals in 5–8 mice. Analysis of cumulative histograms compiled from these data (Cc, Dc) indicated that the I/R-induced reductions in endplate occupancy were significant for both SOD1-G93A/YFP and YFP-only mice ($p < 0.01$ or better, Mann-Whitney 2-tailed test; Kruskal-Wallis test). We found no significant differences in vulnerability to I/R stress among endplates associated with the 4 different heads of the EDL muscle, perhaps because EDL's fiber type composition is quite uniform (98% type IIb or type IIx).

Fig. 2.

In SOD1-G93A/YFP mice, the increased vulnerability of EDL motor terminals to I/R injury begins within the first month; soleus (SOL) motor terminals are more resistant to this stress. Graphs plot mean endplate occupancy as a function of age for YFP-only and SOD1-G93A/YFP EDL (A) and soleus (B) muscles. Filled circles indicate the post-I/R limb; open circles indicate the non-stressed (control) limb. Mean endplate occupancy decreased slowly with age in non-stressed EDLs of SOD1-G93A/YFP mice (dashed line, slope = -0.004 ± 0.0007 , significantly different from zero, linear trend test). In these mice the I/R stress greatly decreased EDL occupancy beginning at ~P30; the exponential fit to these points (solid line) had a time constant of 9.8 days. In contrast, in SOD1-G93A/YFP soleus and in all YFP-only muscles, the slopes of fitted linear regression lines (continuous for stressed, dashed for non-stressed) were not significantly different from zero, indicating maintenance of innervation throughout the sampled age range. In all cases the ischemic stress lasted 30 min; reperfusion times varied from 6–24 hr. Each symbol indicates the mean endplate occupancy computed from 30–80 endplates in the indicated muscle from an individual mouse. Data came from 11 SOD1-G93A/YFP and 16 YFP-only mice aged 24–121 days.

Fig. 3.

Effect of ischemia duration on endplate occupancy in EDL (A) and soleus (B) of SOD1-G93A/YFP and YFP-only mice following I/R. Each data set was fitted with a sigmoidal equation (see Materials and Methods) to calculate the ischemia duration at which mean endplate occupancy would decrease to 50% of maximal (ID_{50}). ID_{50} values ($\pm$ SEM) for EDL were 45 ± 0.5 min in YFP-only vs. 26 ± 1 in SOD1-G93A/YFP; comparable values for soleus were 44 ± 0.4 and 48 ± 1 . All of the following differences were significant at $p < 0.05$ or better (t-test): SOD1-G93A/YFP vs. YFP-only for both EDL and soleus, and YFP-only EDL vs. YFP-only soleus. All curves used maximal and minimal occupancy values of 0.88 and 0, respectively, and a slope of -8.0; the R^2 for all fits exceeded 0.98. Values for 0 min ischemia came from non-stressed muscles. Each point was derived from measurements in 2–14 muscles, except for the 55 min point from a single animal in which the tourniquet was removed after 45 min ischemia, but limb blood flow did not return until 10 min later. More mice were used for ischemia durations that straddled the transitions between maintained innervation and complete denervation, where variability was greatest. In some cases the SEM was smaller than the plotting symbol.

Fig. 4.

Axotomy does not duplicate the effects of I/R on EDL endplate occupancy in a SOD1-G93A/YFP mouse. Cumulative histograms of endplate occupancies were measured in EDL muscles of a P85 mouse in which one hindlimb was subjected to 30 min ischemia followed by 6 hr reperfusion (I/R, filled circles), and the other hindlimb underwent axotomy at the sciatic notch (open circles), which is near the upper hindlimb region where tourniquets were applied. 30–37 endplates were analyzed in each muscle. Comparable measurements for soleus in both axotomized and post-I/R limbs overlapped with those for post-axotomy EDL (not shown).

Fig. 5. SOD1-G93A/YFP EDL motor terminal degenerates rapidly following post-ischemic reperfusion/reoxygenation. Fluorescent micrographs of motor terminals from P76 YFP-only (A) and P69 SOD1-G93A/YFP (B) EDLs that were subjected to 30 min ischemia in vivo. After tourniquet removal, muscles were dissected and placed in physiological saline equilibrated with 95% O_2 /5% CO_2 at 32–34 $^{\circ}\text{C}$. Images taken 35 min and 100 min following conclusion of the ischemic stress show that the YFP-only terminal remained intact (Aa,b), whereas the SOD1-G93A/YFP terminal broke apart (Ba,b). Dotted lines in Ba outline the preterminal axon at 35 min; this region had disappeared by 100 min (Bb). Arrowheads in B indicate a section of the terminal that was continuous at 35 min, but became fragmented by 100 min. Bc, filled circles show the time course of fluorescence decay in the SOD1-G93A/YFP terminal; open circles show that the fluorescence of a neighboring background region did not change. The plot begins at 30 min, the time required to dissect and mount the muscle. Note that this graph

measures total YFP fluorescence (arbitrary units); YFP measurements made for all other figures included only those sections of the motor terminal that remained connected to the preterminal axon. Similar results were obtained in 2 additional post-I/R SOD1-G93A/YFP terminals and 3 additional post-I/R YFP-only terminals.

Fig. 6. EDL endplates in a SOD1-G93A/YFP mouse are reinnervated 10 days following a 30 min I/R stress. Micrographs are overlays of α -BgTx-labelled endplates (red) and YFP-labelled motor axons (green) from non-stressed (left) and stressed (right) hindlimb. Ischemia was applied at P65. Arrowheads indicate terminal sprouting that extended beyond labelled endplates.