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Functional over-load saves motor units in the SOD1-G93A transgenic mouse model of amyotrophic lateral sclerosis

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ABSTRACT

The fastest, most forceful motor units are lost progressively during asymptomatic disease in the SOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis. As the disease progresses the surviving motor units must increase their levels of activity to sustain posture and movement. If activity-dependent conversion of motor units to more fatigue resistant types increased their resilience and hence survival, we hypothesized that an experimental increase in motor unit activity in the hindlimb muscles of the SOD1^{G93A} transgenic mouse should "save" those motor units that are normally lost in the first 90 days of age. To test this hypothesis, we partially denervated hindlimb muscles in SOD1^{G93A} and their corresponding control SOD1^{WT} transgenic mice by avulsion of either L4 or L5 spinal roots at 40 days of age. Whole muscle and single motor unit isometric twitch forces were recorded and the numbers intact motor units in fast-twitch tibialis anterior, medial gastrocnemius, extensor digitorum longus muscles and the slow-twitch soleus muscle were calculated at 90 days of age. We found that the rapid age-dependent decline in numbers of functional motor units in fast-twitch muscles of the SOD1^{G93A} transgenic mice was dramatically reduced by the functional hyperactivity in the partially denervated muscles and, that these muscles comprised a significantly higher component of type IIA and type IID/X fibers than those muscles that were innervated by nerves in intact spinal roots. We conclude that the vulnerable motor units are saved by increasing their neuromuscular activity and consequently, converting them to slower, less forceful, fatigue resistant motor units.

Introduction

Progressive motoneuron loss in amyotrophic lateral sclerosis (ALS) results in skeletal muscle denervation, manifesting as weakness and eventual paralysis. A mutation within the gene encoding the antioxidant enzyme cytosolic copper/zinc superoxide dismutase (SOD1) is associated with ~20% of familial ALS cases (Rosen, 1993). The SOD1^{G93A} transgenic mouse expressing the most common human mutation of a glycine to alanine conversion at the 93rd codon of SOD1 in high copy number develops symptoms and pathology similar to ALS patients (Gurney et al., 1994). The mechanisms of toxicity of the mutation remain unknown but studies of transgenic mouse lines indicate that mutant SOD1 toxicity is not related to copper-mediated catalysis of the conversion of toxic superoxide radicals to hydrogen peroxide and oxygen (Cleveland, 1999; Boillee et al., 2006). Proposed mechanisms include first, a gain of function for mSOD1 with reduced zinc binding to transform the protein into a toxic pro-oxidant and second, a propensity of a subfraction of mutant SOD1 proteins to form misfolded proteins and aggregates that saturate chaperones, inhibit proteasomes and/or interact with mitochondrial proteins (Rowland and Shneider, 2001; Boillee et al., 2006; Julien and Kriz, 2006; Rakhit et al., 2007).

Each motoneuron normally innervates tens and even thousands of muscle fibers, the neuron and its muscle fibers commonly referred to as the motor unit (MU) (Gordon et al., 2004b). Motoneurons are lost in a "die-back" manner with muscle denervation and loss of functionally intact MUs preceding loss of the axons, thinning of nerves in ventral roots, and motoneuron loss from the spinal cord (Chiu et al., 1995; Frey et al., 2000; Fischer et al., 2004; Hegedus et al., 2007; Gordon et al., 2008; Parkhouse et al., 2008). Die-back preferentially affects large motoneurons that innervate the most forceful type IIB muscle fibers (Henneman et al., 1965; Henneman and Olson, 1965; Frey et al., 2000; Pun et al., 2006; Hegedus et al., 2008), in accordance with selective vulnerability of large motor axons in sporadic ALS (Kawamura et al., 1981). Selective retardation of slow axonal transport of neurofilaments and tubulin in ALS mouse models that commonly occurs before detectable pathology results in their cellular accumulation. This may be the critical event leading to

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progressive strangulation of the nerves, the largest being the most vulnerable (Williamson and Cleveland, 1999).

Cellular mechanisms underlying the rapid preferential decline of the largest motor units during the asymptomatic phase in ALS are not yet understood. Dramatic loss of the most forceful MUs with the largest axons in the SOD1^{G93A} transgenic mice necessitates increased recruitment of surviving MUs and thereby, increased neuromuscular activity of the remaining pool of motoneurons and their muscle fibers (i.e. the MUs). This increased neuromuscular activity may, in turn, account for conversion of muscle fibers from IID/X to IIA phenotypes that is already apparent at 60 days of age in the TA muscle of SOD1^{G93A} mice (Hegedus et al., 2007; Hegedus et al., 2008). The motoneurons innervating type IIB and IID/X muscle fibers are the most susceptible to die-back and increased neuromuscular activity. The associated conversion of MU phenotypes with concurrent size reduction may confer some protection owing to reduced conduction velocity and axonal size (Munson et al., 1997; Havton et al., 2001). In the present study, we test the hypothesis that increased neuromuscular activity in the SOD1^{G93A} transgenic mouse model of ALS prolongs functional contact of motor axons with their skeletal muscles and promotes the retention of functional MUs. In order to increase neuromuscular activity of hindlimb muscles of the SOD1^{G93A} transgenic mouse, we used the physiological method of functional overload of hindlimb muscles by partial denervation of the hindlimbs after avulsing one of two contributing spinal roots.

Methods

Generation of SOD1^{G93A} mice

Male transgenic mice that express mutant human SOD1^{G93A} genes (B6JSL-TgN (SOD1-G93A) were purchased from Jackson Laboratories, USA. A colony was established by breeding male SOD1^{G93A} transgenic mice to non-transgenic B6JSL female mice. We identified transgenic SOD1^{G93A} mice from the offspring of these matings through standard PCR protocol for the human SOD1 performed on ear samples taken at the time of weaning (Rosen, 1993). After the pups were weaned at ~21 days of age they were separated by gender into standard rodent cages with free access to food and water. All of the experiments were approved by the University of Alberta Health Sciences Laboratory Animal Ethics committee and were carried out in accordance with the guidelines of the Canadian Council for Animal Care.

Partial denervation of hindlimb muscles

We partially denervated one hindlimb in 32 male mice (22 SOD1^{G93A} transgenic mice and 10 age-matched wild-type SOD1^{wt} control mice) at 40 days of age. Partial denervation was completed by avulsing one of two spinal roots that innervates the hindlimbs. Mice were anaesthetized by an intraperitoneal (IP) injection of an anesthetic cocktail made up of ketamine (100 mg/ml) and atravet

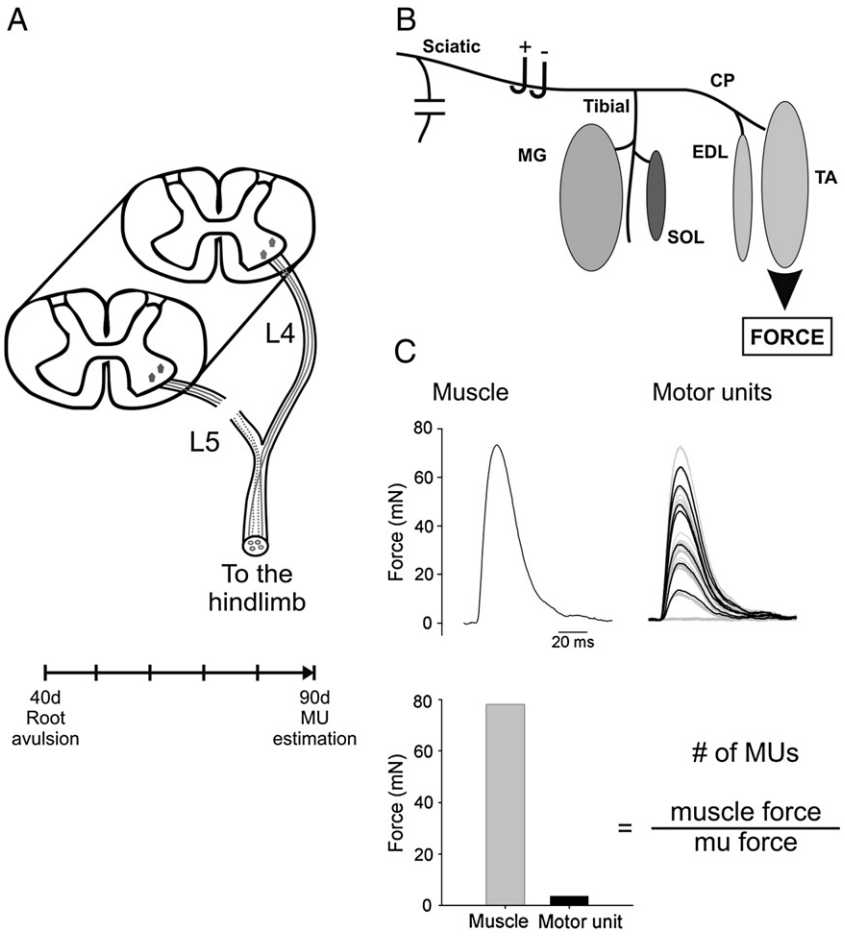

Fig. 1. (A) At 40 days of age, the muscles in one hindlimb were partially denervated by cutting one of two primary spinal roots, L4 and L5. The proximal nerve stump of the root was grasped with forceps to avulse the root, thereby preventing axon regeneration. (B) The tendons of the fast-twitch MG and slow-twitch SOL muscles of the ankle extensor group and those of the fast-twitch TA and EDL muscles of the ankle flexor group were isolated to connect to a force transducer to measure isometric contractions in response to stimulation of the sciatic nerve. (C) Isometric force was measured in response to maximum stimulation of the sciatic nerve at 1 Hz to elicit twitch contractions and to 100 Hz to elicit maximal tetanic contractions (not shown). Incremental stimulus voltage on the sciatic nerve elicited all-or-none increases in contractile force. Using a method to randomly select 15 levels of force in response to these increments, the number of motor units was estimated (MUNE) by division of the muscle and the average motor unit force. Details in the text.

(10 mg/ml) diluted in sterile saline. The dosage of the anesthetic was 17.5 ml/kg body weight for both control and $SOD1^{G93A}$ mice.

A small incision was made along the spinal cord just above the iliac crest to expose the nerves that radiate from the spinal roots. From the iliac crest, we counted the spinal roots to identify the nerves coming from the L4 and the L5 segments on the right side of the mouse (Fig. 1A). Using fine forceps and scissors, the bone around the segment of interest was cleared of muscle, and the proximal nerve stump of either the L4 or the L5 spinal root was firmly grasped and avulsed to prevent axon regeneration. The skin incision was sutured closed and the mice were allowed to recover before being returned to their cages. For the next 50 days, all experimental mice were subject to daily health checks during which their general health and mobility were evaluated.

Electrophysiological recordings

Surgery

The method used to enumerate and characterize motor units (MUs) has been described in detail (Major et al., 2007; Hegedus et al., 2009a). In brief, mice were anesthetized with the ketamine/atravet cocktail injected IP to induce surgical anesthesia. Periodically, additional doses of the cocktail were administered IP in order to maintain anesthesia throughout the experiment. The lower hindlimbs of the mice were exposed, and the tendons to the plantaris and lateral gastrocnemius muscles were identified and cut. Tendons to the soleus (SOL), medial gastrocnemius (MG), tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were identified and separated. These muscles were tied individually with a 4.0 silk thread for attachment to the strain gauge (Kulite model KH-102) during recording (Fig. 1B). Following isolation of the nerves bilaterally, the sciatic nerves were exposed on both sides and wire electrodes were sutured alongside the nerves for electrical stimulation. Both hindlimbs were prepared in all mice and the non-operated side was used as a control. The hindlimbs were clamped at the knees and ankles to immobilize the legs without interfering with the blood supply to the muscles.

Isometric force recordings and MU enumeration

Isometric forces evoked from individual muscles through electrical stimulation of the sciatic nerve were amplified and digitized using Axoscope Software (version 8.0, Axon Instruments, USA). The lengths of the muscles were adjusted to yield maximal evoked isometric twitch contractile force in response to stimulation of the sciatic nerve. Maximal contractile muscle forces were recorded in response to single suprathreshold (2× threshold amplitude) stimulation of the sciatic nerve at a frequency of 0.5 Hz.

Average MU force was determined by incremental stimulation of the sciatic nerve to elicit discrete increases in MU force as described in detail previously and illustrated in Fig. 1C (Hegedus et al., 2007; Major et al., 2007; Hegedus et al., 2009a). The incremental increases in whole muscle force were recorded in response to sciatic nerve stimulation at a frequency of 0.5 Hz, and the amplitude of the stimulus pulse was manually controlled from 0 to 10 V. The all-or-none increments in muscle force were recorded and overlaid in a custom software program written in MatLab. Using this program, 8–20 increments were randomly chosen from throughout the entire range of muscle forces. We calculated average MU force as the mean force associated with these increments. In order to estimate the number of intact MUs, the whole muscle twitch contractile force was divided by the average MU force.

Determination of muscle fiber composition by SDS-PAGE and histochemistry

Myosin heavy chain (MHC) isoforms were analyzed according to Gallo et al. (2004) Briefly, muscles from both SOD^{G93A} and SOD^{WT} transgenic mice were homogenized on ice with buffer containing 100 mM $Na_4P_2O_7$ (pH 8.5), 5 mM EGTA, 5 mM $MgCl_2$, 0.3 M KCl, 10 mM DTT and protease inhibitor cocktail at 5 mg/ml concentration (CompleteTM, Roche Diagnostic, Laval, PQ, Canada). Samples were subsequently stirred for 30 min on ice followed by centrifugation at 13,400×*g* for 5 min at 4 °C. The supernatant of each sample was then isolated and diluted 1:1 with glycerol and stored at -20 °C until analyzed. Extracts were diluted to 0.1 μg/μl in modified Laemmli lysis buffer, boiled for 6 min, and cleared by centrifugation. The MHC extract in a volume of 6 μl was electrophoresed in triplicate for 24 h at constant 275 V and 12 °C on polyacrylamide gels containing glycerol. Gels were then fixed and MHC isoforms were detected by silver staining (Oakley et al., 1980). The relative proportions of MHC isoforms were determined with integrative densitometry (Syngene ChemiGenius, GeneTools, Syngene, Cambridge, UK).

Statistics

Data were considered significant if $p<0.05$, and p was determined using an independent samples t-test (SPSS version 14.0, 2005). Significance is denoted by stars in the figures (* for $p<0.05$ and ** for <0.01). For calculations including MU forces we tested the data set for normality using a one-sided Kolmogrov–Smirnov test. For non-normally distributed data, significance was determined using the Mann–Whitney U-test in the SPSS program.

Results

Many functional motor units are lost in fast-twitch muscles of $SOD1^{G93A}$ mice in the pre-symptomatic phase of disease

Functional motor units (MUs) are the motoneurons and the muscle fibers that they innervate. By isolating axons for stimulation,

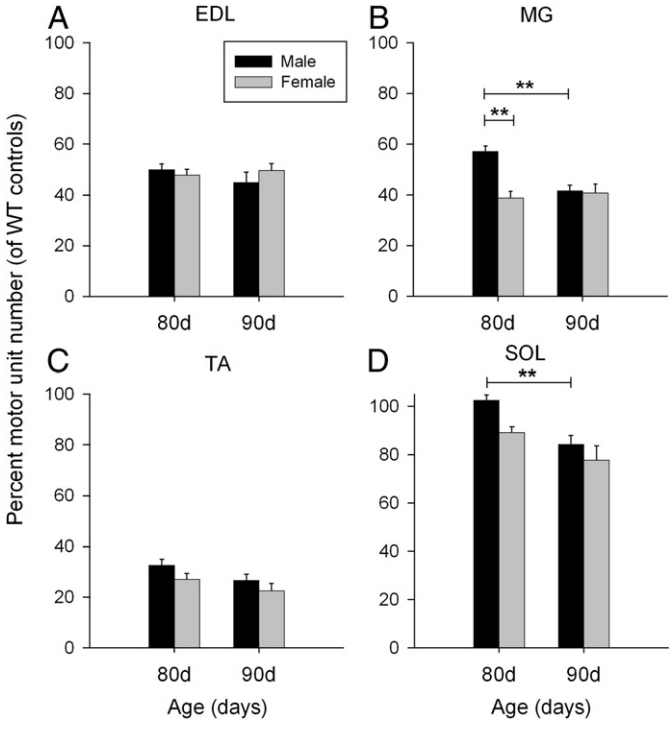

Fig. 2. The number of motor units in fast-twitch EDL, MG and TA muscles and slow-twitch SOL muscles in $SOD1^{G93A}$ transgenic mice at 80 and 90 days of age in male and female mice. The number of motor units is expressed as a percent of the number in $SOD1^{WT}$ transgenic mice. Although there was a significant decline in motor unit numbers between 80 and 90 days of age in the SOL muscle there was only a significant reduction in the MG muscle for the male mice. This gender difference not being significant over time (Hegedus et al., 2009b), both genders were used in the remaining studies of partial denervation. $^{**}p<0.01$ and $^{*}p<0.05$.

we recorded the isometric force of single MUs in normally caged SOD1^{G93A} transgenic mice. As illustrated in Figs. 1B, C, the isometric contractile force of the fast-twitch medial gastrocnemius (MG), extensor digitorum longus (EDL) and tibialis anterior (TA) muscles and the slow-twitch soleus (SOL) muscles was recorded in turn in each animal. At a frequency of 1 Hz, supramaximal stimulation of the sciatic nerve to the muscles evoked muscle twitch contractile force. Using incremental stimulation described in the Methods section, we recorded forces developed by the muscle fibers innervated by single motoneurons–single MUs. The ratio of the muscle and the mean MU twitch contractile forces provided the estimation of the number of intact MUs.

We found that the numbers of MUs rapidly declined from 40 days of age in the fast-twitch hindlimb muscles, reaching a plateau between 80 and 90 days when the mice began to show symptoms of hindlimb weakness at the onset of symptomatic disease (Chiu et al., 1995; Veldink et al., 2003; Hegedus et al., 2007). At 80 days of age, a large percentage of the intact MUs innervating the fast-twitch hindlimb muscles were already lost ($n=5$ for males, $n=8$ for females; Fig. 2). In the EDL and MG muscles, ~50% of the functional MUs remained at 80 and 90 days of age; although the percentage of functionally intact MUs that remained was significantly higher in the MG muscles in the male as compared to the female SOD1^{G93A} mice ($P<0.01$) (Figs. 2A, B), no systematic gender difference was seen during the progressive decline in MU numbers over the course of the ~140 day lifespan of the mice (Hegedus et al., 2009b). In the TA muscles of the 80 to 90 day old SOD1^{G93A} transgenic mice only ~25% of the functional MUs remained (Fig. 2C). The decline of functional MUs in the slow-twitch SOL muscle occurred later with a significant decline after 80 days of age (Fig. 2D). This is consistent with the relative saving of the type I muscle fibers during the asymptomatic phase of the disease (Gordon et al., 2005; Hegedus et al., 2007; Hegedus et al., 2009b).

Partially denervated muscles in SOD^{wt} transgenic mice enlarge their motor units to compensate for loss of intact motor units

The spinal roots that emanate from the L4 and L5 segments of the spinal cord in the mouse innervate the ankle extensor and flexor muscles of the hindlimb. Either the L4 or L5 spinal root was avulsed unilaterally at 40 days of age in SOD1^{G93A} and SOD^{wt} transgenic mice of both male and female mice to partially denervate the hindlimb muscles. Thereby neuromuscular activity in the remaining intact MUs is increased to sustain posture and movement (Einsiedel and Luff, 1994; Seburn and Gardiner, 1996; Tyc and Vrbova, 2007). In human patients with neurogenic lesions the maximal discharge rate of MUs increases as the number of MUs in the muscles decreases (Schulte-Mattler et al., 2000). Because the MU loss was the same in male and female SOD1^{G93A} transgenic mice (Hegedus et al., 2007; Hegedus et al., 2009b), our further analyses of partially denervated muscles were performed on transgenic mice of both genders.

Avulsion of the L4 spinal root in the SOD^{wt} transgenic mice reduced the mean number of intact MUs in the TA muscle from 77 to 30, a significant reduction of about 60% (Fig. 3A). Based on evidence in several animal species including cat, rat and mouse, and humans, loss of up to 80% of MUs is normally compensated for by collateral sprouting in the order of 3–8 fold to enlarge remaining intact MUs (Yang et al., 1990; Rafuse et al., 1992; Gordon et al., 1993; Tam et al., 2001; Tam and Gordon, 2003a; Gordon et al., 2004a). Collateral sprouting and reinnervation in the SOD^{wt} mice was evident as increased MU forces and preservation of total muscle contractile force even after avulsion of the L4 spinal root. Fig. 3 demonstrates a significant rightward shift of the MU force histograms to larger MU forces at 90 days of age (Fig. 3B) with a significant increase in their average MU force (Fig. 3C). Recovery of muscle twitch force was equal that in the contralateral muscles where the innervation remained intact (Fig. 3D).

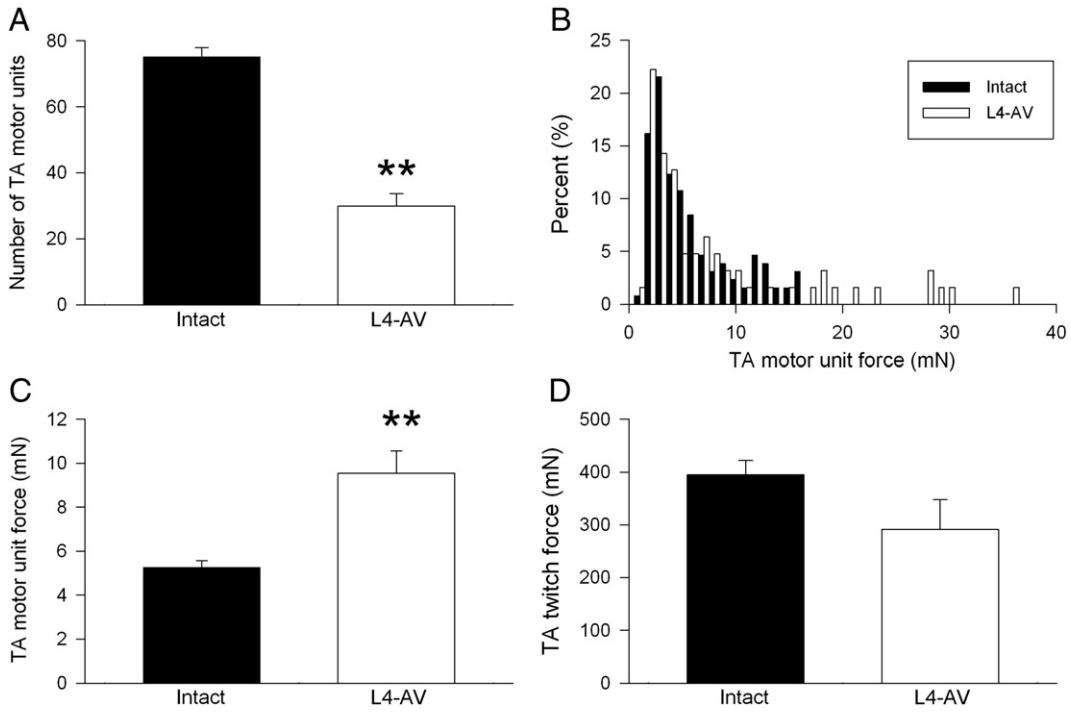

Fig. 3. The response of TA muscle in the SOD1^{WT} transgenic mouse to partial denervation by avulsion of the L4 spinal root (L4-AV). (A) The number of motor units was significantly reduced in the partially denervated TA ($p<0.01$) with a significant shift of the percentage frequency histogram (B) of motor unit forces to the right. The average increase in TA motor unit force (C) was significant and corresponded with a similar decline in numbers of intact motor units remaining in the partially denervated TA muscle. (D) As a result of the motor unit enlargement, the partially denervated TA muscle after L4 avulsion recovered whole muscle twitch force that was not significantly different from the intact contralateral TA muscle. **$p<0.01$ and *$p<0.05$.

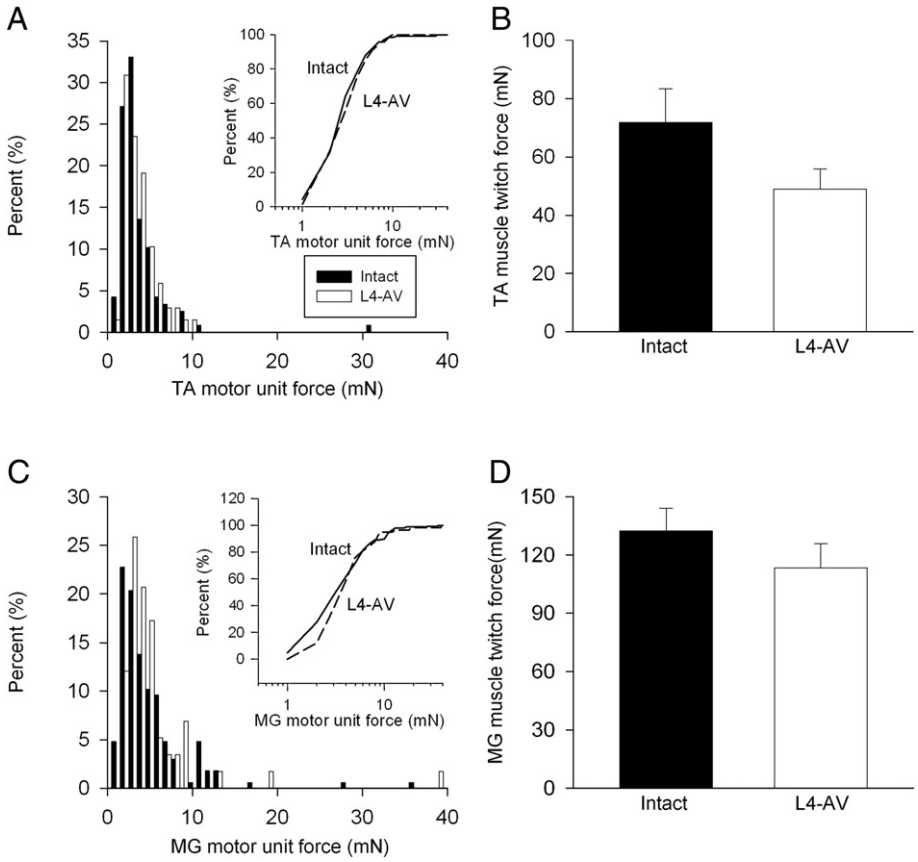

Fig. 4. Little or no enlargement of motor unit twitch contractile forces after partial denervation of the TA and MG muscles in the hindlimbs of SOD1^{G93A} transgenic mouse *but* full recovery of muscle forces despite avulsion of the L4 spinal root. In the TA (A) and MG (C) muscle there was no significant shift of the percentage frequency histograms and of the cumulative percent histogram of motor unit forces to the right, despite the significant reduction in the numbers of remaining motor units at 40 days of age that was shown in Fig. 3 and Supplementary Fig 1. Yet the muscles developed as much twitch force in response to maximal stimulation of the sciatic nerve as did the contralateral muscles that developed force in response to stimulation of *all* the motor nerves emanating from spinal roots L4 and L5 (B, D). The findings indicate that the motor units that remained after L4 spinal root avulsion survived during the next 50 days until force recording at 90 days of age.

Avulsion of L4 spinal root also reduced numbers of intact MUs in the MG muscle (Supplementary Fig. 1). Similar to the TA muscle, the force frequency histograms for the partially denervated MG muscle were shifted to the right with increased MU forces. The increased mean forces compensated for the reduced number of MUs, resulting in comparable contractile muscle force in the partially denervated and intact muscles (Supplementary Fig. 1). In contrast no significant reduction was found in SOL muscles whose motor axons exit the spinal cord in the L5 spinal root (see Fig. 5D). The EDL muscle which is similar in muscle fiber type composition as compared to the synergistic TA muscle demonstrated a similar progression and rate of MU loss. The muscle not being significantly denervated by either L4 or L5 was not studied further.

Partially denervated muscles in SOD1^{G93A} transgenic mice do NOT enlarge their surviving motor units but they develop as much force as their contralateral intact muscles

The L4 spinal root was avulsed in the SOD1^{G93A} transgenic mice, reducing the number of MUs in the muscles by the same proportion as in the SOD^{wt} mice. However, the increased MU twitch forces seen in the TA and MG muscles in the SOD^{wt} mice were *not* found in the SOD1^{G93A} transgenic mice (cf. Figs. 4 and 3, Supplementary Fig. 1). There was little or no shift of the force histograms to the right in the 90 day old SOD1^{G93A} mice after partial denervation of the muscles at 40 days of age (Figs. 4A, C). There was, however, a trend for the smaller MUs to enlarge in the MG muscles (insert in Fig. 4C) where the proportion of type I and IIA muscle fibers are higher than in the TA muscle, consistent with the known capacity of motor nerves innervating these fibers to sprout in the SOD1^{G93A} transgenic mouse muscles, unlike those innervating the larger muscle fibers (Frey et al., 2000).

Despite there being no or minimal MU enlargement, after partial denervation of the hindlimb of the SOD1^{G93A} transgenic mice, the contractile force in the partially denervated muscles was the same as that in the muscles in the contralateral intact hindlimb (Figs. 4B, D). Since muscle contractile force is the product of the number of MUs in the muscle and the force they develop (Tötösy de Zepetnek et al., 1992) and the MU forces in the partially denervated and intact muscles were not significantly different (Figs. 4A, C), we conclude that the number of MUs in fast-twitch muscles that normally decline sharply from 40 to 90 days of age (see Fig. 6) do not, all the MUs in the partially denervated muscles surviving. Thus the normal muscle contractile forces in the partially denervated TA (Figs. 3D and 5B) and MG muscles (Supplementary Fig. 1, Fig. 4D) indicate that the remaining MUs after avulsion of one spinal root, remain intact and do *not* decline in number over the 40 to 90 days as those MUs that do decline in number in the intact contralateral limb of the SOD1^{G93A} transgenic mouse (see Figs. 5–7).

Partial denervation by avulsion of the L5 spinal root produced similar results to avulsion of the L4 spinal root. L5 avulsion resulted in a significant loss of functional MUs in both the fast-twitch TA and MG muscles and the slow-twitch SOL muscle in SOD1^{G93A} transgenic and SOD^{wt} mice. Data for the TA muscle is shown in Figs. 5A–C and data for MG and SOL muscles are shown in Supplementary Figs. 2C,F. Sprouting and reinnervation in the partially denervated SOD^{wt} mice resulted in a significant rightward shift of the MU force distributions to larger forces and increases in mean MU force compared to the

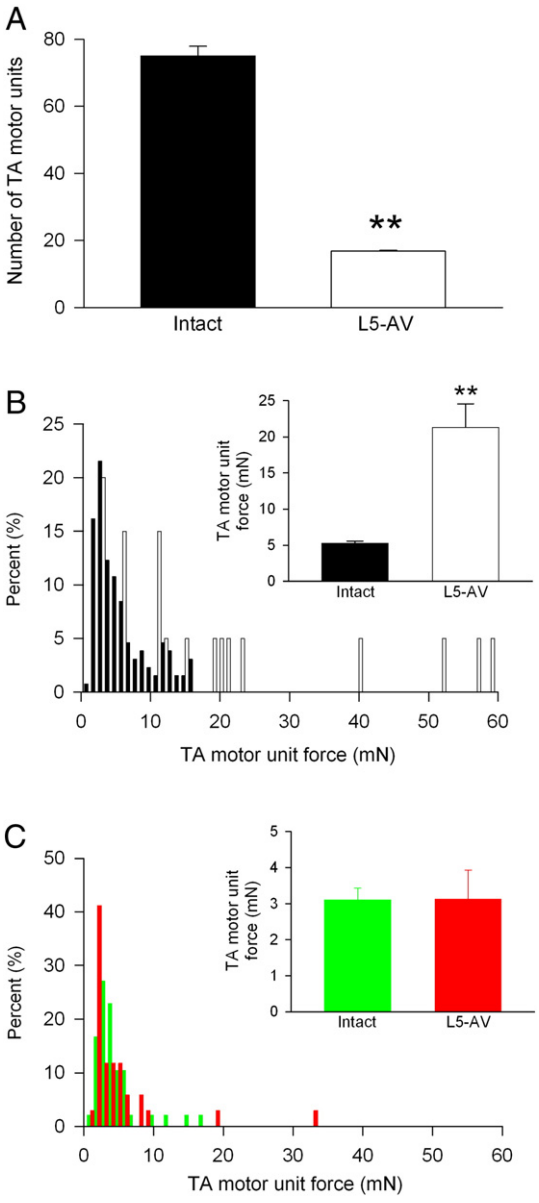

Fig. 5. Little or no enlargement of motor unit twitch contractile forces after partial denervation of the TA muscle in the hindlimbs of SOD1^{G93A} transgenic mouse following avulsion of the L5 spinal root. Avulsion of L5 spinal root significantly reduced the number of motor units in the fast-twitch TA muscle (A). The percentage frequency histograms and the cumulative percent histograms of motor unit forces in the SOD1^{WT} transgenic mice but *not* the SOD1^{G93A} transgenic mice were shifted significantly to the right for the TA muscle (cf. C with B) ($P<0.01$). **$p<0.01$ and *$p<0.05$.

intact contralateral muscles (Fig. 5B; Supplementary Fig. 2B). In contrast MU force distributions and mean MU force in SOD1^{G93A} transgenic mice were the same for the partially denervated and intact contralateral muscles (Fig. 5C; Supplementary Fig. 2C,F). The data are consistent with our previous findings that TA MU forces in the 60 day old SOD1^{G93A} mouse did not increase significantly to compensate for the age-dependent decline of ~70% in the proportion of intact MUs in the SOD1^{G93A} transgenic mice relative to the SOD^{wt} mice (Hegedus et al., 2007). While there was a trend in that study for a similar increase in MU force, the change was not significant. Since only the motoneurons that supply the small MUs with type I and IIA muscle fibers sustain their sprouting capacity in the SOD1^{G93A} transgenic mouse (Frey et al., 2000; De Winter et al., 2006; Pun et al., 2006), any enlargement of the small proportion of low force MUs with type I and IIA muscle fibers was not detected as reported earlier (Hegedus et al., 2008).

More intact motor units survive in the more active partially denervated muscles of the SOD1^{G93A} mouse to account for the sustained muscle force in the muscles

During the asymptomatic phase of disease prior to 90 days of age, there is a clear decline with age in numbers of intact MUs in the fast-twitch hindlimb muscles of SOD1^{G93A} transgenic mice (black symbols, Figs. 6A, C, E), when compared with the number of MUs in the SOD1^{WT} transgenic mice. Avulsion of the L5 spinal root removed at least 50% of the motor axons that normally innervate the TA, MG and SOL muscles in both SOD^{wt} and SOD1^{G93A} transgenic mice (Figs. 6A, C, E). Given this reduction, it follows that the predicted decline in surviving MUs in the partially denervated muscles of the SOD1^{G93A} transgenic mice should parallel the decline with age seen for each muscle in the hindlimbs supplied by intact spinal roots (gray symbols, Figs. 6A, C, E). The *actual* number of MUs counted in the muscles at 90 days of age after the L5 spinal root avulsion (at 40 days of age) was much greater than the predicted number after their age-dependent decline (open symbols, Figs. 6A, C, E). Indeed, the number of functional MUs after L5 root avulsion was the same as the number for the TA and MG muscles in the hindlimb supplied by both L4 and L5 in the unoperated contralateral hindlimb (Figs. 6B, D). In the SOL muscle, where the reduction in MU numbers after L5 root avulsion was ~35%, the numbers of MUs innervating the partially denervated muscles in the SOD^{G93A} mouse were still greater than predicted. However, the numbers were not as high as in the 90 day old muscles of hindlimbs with intact spinal roots (Fig. 6F). The significant reduction in MU numbers in muscles of the SOD^{wt} mice after L5 root avulsion contrasts with the survival of all MUs in muscles of the SOD1^{G93A} transgenic mice after the L5 avulsion days earlier (Fig. 7).

An additional comparison was made, as shown in Fig. 8 and Supplementary Fig. 3 between MU numbers expected (predicted) after the L5 root avulsion (in gray), and the actual numbers counted in the partially denervated muscles relative to the mean numbers of MUs in the SOD1^{G93A} transgenic mice with intact spinal roots. It is clear that the functional sequelae of the partial denervation of the muscles at 40 days of age sustained the survival of all the MUs that remained (Fig. 8A; Supplementary Fig. 3A,D). It was the sustained numbers of intact MUs, and not an increased force capacity of those MUs (Fig. 8B; Supplementary Fig. 3B,E) that accounted for the contractile forces of the partially denervated muscles equaling the forces of the contralateral muscles whose spinal root innervation remained intact (Fig. 8C; Supplementary Fig. 3C,F).

Partially denervated muscles of the SOD1^{G93A} mouse exhibit phenotypes of more aerobic slower muscle fibers

The selective vulnerability of MUs that contain the type IIB muscle fibers during the asymptomatic phase of disease in SOD^{G93A} transgenic mice was accompanied by increased proportions of type IIA muscle fibers but not with elimination of *all* the type IIB muscle fibers (Hegedus et al., 2007; Hegedus et al., 2008) as was previously claimed by Frey et al. (2000), De Winter et al. (2006), and Pun et al. (2006). The fiber type transition observed in TA muscles of the 60 day old SOD^{G93A} transgenic mice suggested that the increased neuromuscular activity of the surviving MUs accounted for the conversion and, perhaps, in turn, the survival of the active MUs (Hegedus et al., 2008). We asked whether the survival of all intact MUs in partially denervated muscles of the 90 day old SOD1^{G93A} transgenic mouse was accompanied by increased conversion of muscle fibers to more oxidative phenotype.

Examination of the partially denervated TA and MG muscles in the SOD^{G93A} mice showed that, indeed, the muscle fibers at 90 days of age had undergone significant transformation from predominantly type IIB and type IID/X fibers toward increased proportions of type IIA (type IIA are commonly referred to as fast-oxidative fibers) (Pette and Vrbova, 1999). These muscles also displayed increased expression of

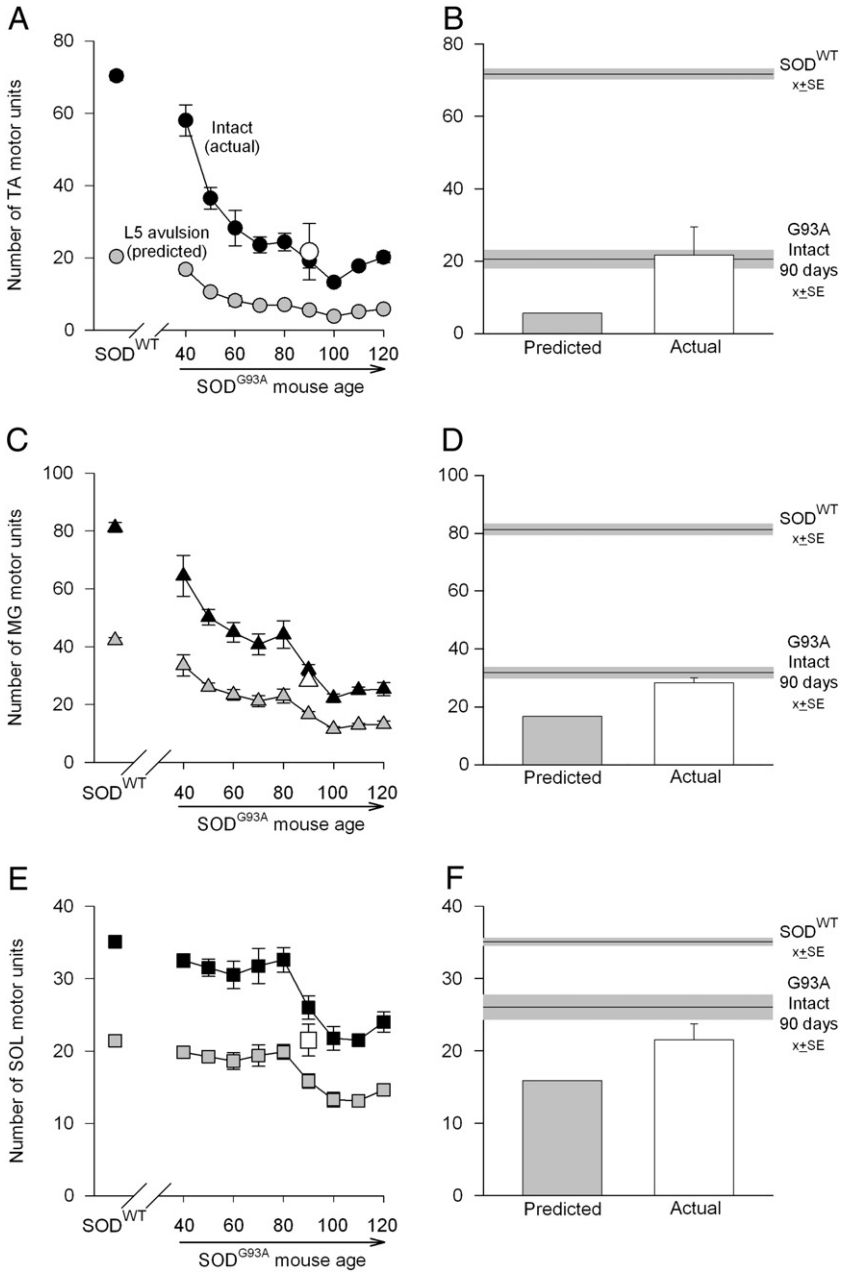

Fig. 6. The mean (+SE) numbers of motor units in partially denervated TA (A, B), MG (C, D) and SOL (E, F) muscles at 90 days of age after L5 root avulsion in SOD1^{G93A} transgenic mice, are compared with the numbers in the mice with intact spinal roots. The reduced number of motor units resulting from avulsion of L5 spinal root is shown for the SOD1^{WT} transgenic mice with the predicted number of motor units in the SOD1^{G93A} transgenic mice plotted as a function of age and compared directly with the mean (+SE) numbers of motor units (A, C, E). The histograms compare the actual motor unit numbers for the muscles with the predicted numbers and demonstrate that for the fast-twitch TA (B) and MG (D) muscles and for the slow-twitch SOL muscle (F), the actual numbers are not significantly different from the numbers recorded in muscles of the SOD1^{G93A} transgenic mice in which all spinal roots were intact (B, D, F). In panels (A, C, E) black symbols represent actual measurements, gray represent predicted measures assuming the same rate of degeneration following partial denervation, and the open symbols represent the actual values measured in partially denervated muscles at 90 days of age.

the slowest MHC-I isoform after L4 spinal root avulsion (Fig. 9). By comparison, expression of MHC-I and MHC-IIA in the EDL muscles also increased, as the fast IID/X and IIB fibers transitioned toward these slower phenotypes (Fig. 9). A trend for an increase in MHC-I were observed in the SOL muscle ($p = 0.06$), probably as this slower isoform was expressed in the IIA fibers.

Discussion

Our major finding was that increased neuromuscular activity prevents the precipitous loss of motor units (MUs) from hindlimb muscles that normally occurs during presymptomatic disease in a transgenic mouse model of ALS. Functional overload of one hindlimb in the SOD1^{G93A} mouse by cutting one of two contributing spinal roots at 40 days of age, results in survival of *all* functional MUs at 90 days as compared to ~30–60% survival without root avulsion. This supports our hypothesis that increased neuromuscular activity "saves" functionally intact MUs. Partial denervation as the model of increased neuromuscular activity, resulted in muscle fiber conversion from type IIB through IID/X to type IIA and I, providing support that these muscles experienced increased MU recruitment.

The findings are consistent with fiber conversion to more oxidative phenotype during the rapid attrition of forceful fast MUs in the SOD1^{G93A} transgenic mouse; lumbosacral motoneurons innervating the fatigable type IIB muscle fibers die-back during asymptomatic disease (Hegedus et al., 2007; Hegedus et al., 2008). Coincident

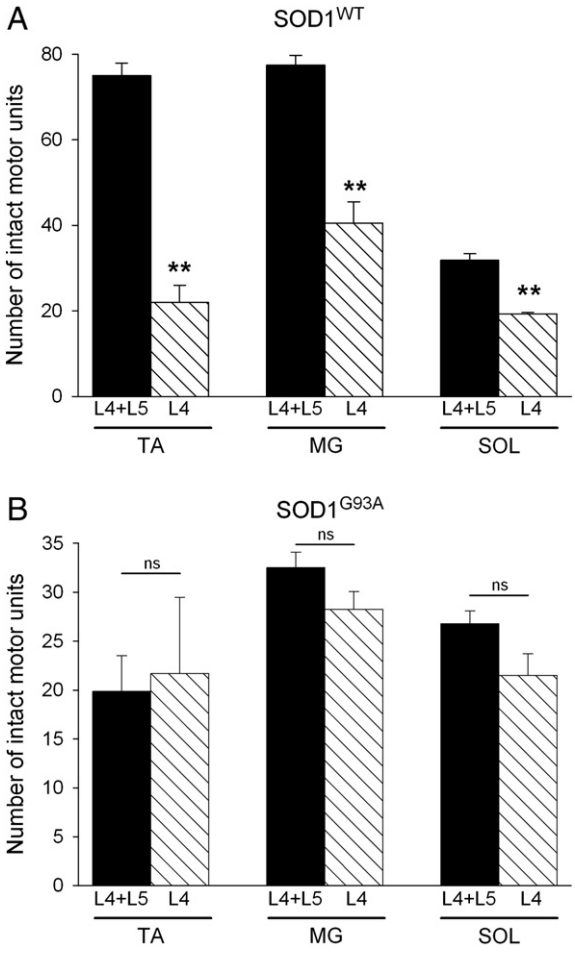

Fig. 7. Comparison of the mean (+SE) number of intact motor units and those remaining in the partially denervated muscles after cutting the L5 spinal root in hindlimb muscles in SOD1^{WT} (A) and the SOD1^{G93A} (B) transgenic mice. **$p<0.01$ and *$p<0.05$.

conversion towards slow phenotype suggested the possibility that the conversion is a positive adaptive response of reduced MU numbers to their increased neuromuscular activity. Indeed, MU numbers plateau during symptomatic disease after 90 days of age (Hegedus et al., 2007) again indicating that increased recruitment of fewer functional MUs is adaptive for survival. Indications from our time course data with the contrasting sensitivity of fast-twitch as compared to slow-twitch muscles were confirmed and expanded by our present findings of parallel conversion of muscle fiber types and 'saving' of the motoneurons innervating more fatigue resistant muscle fibers.

A proportion of muscle fibers is denervated after cutting one ventral root and intramuscular axons of the remaining intact MUs normally sprout to reinnervate denervated muscle fibers: perisynaptic Schwann cells extend processes that bridge between endplates that lead axonal sprouts to innervate denervated endplates (Son and Thompson, 1995a; Son and Thompson, 1995b; Love et al., 2003; Tam and Gordon, 2003a; Gordon et al., 2004a). Sprouting enlarges MUs by including more muscle fibers per motoneuron and in turn, increases their force output (Rafuse et al., 1992; Gordon et al., 1993; Fu and Gordon, 1995; Rafuse and Gordon, 1996a; Rafuse and Gordon, 1996b; Tam and Gordon, 2003a; Gordon et al., 2004a). The observed increased MU forces in the partially denervated muscles of the SOD^{wt} transgenic mice were directly proportional to the reductions of up to 80% in MU numbers, consistent with previous reports of MU enlargement in rodents (Havton et al., 2001; Tam and Gordon, 2003b; Gordon et al., 2004a). The sprouted axons reinnervate all denervated fibers to fully restore muscle twitch and tetanic contractions to normal levels. MU enlargement in the SOD^{wt} transgenic mice was seen in the large MG and TA muscles that were partially denervated by >50% in contrast to the SOL and EDL muscles where the resolution for MU enlargement was insufficient to detect significant increases in MU forces for <50% partial denervation (Rafuse et al., 1992).

MU enlargement was minimal in the partially denervated muscles in the SOD1^{G93A} transgenic mice in contrast to the SOD1^{WT} transgenic mice. This is consistent with immunocytochemical evidence that motor nerves innervating type IIB muscle fibers fail to sprout in the presence of the chemorepellant semaphorin 3A in the terminal Schwann cells at muscle endplates of type IIB fibers of SOD1^{G93A} transgenic mice (Frey et al., 2000; De Winter et al., 2006). Evidence of MU enlargement of less forceful MUs in fast-twitch TA and MG muscles indicated some degree of effective sprouting of the motor nerves supplying type I and IIA muscle fibers. Muscle force is the product of the number and contractile force of its component MUs (Tötösy de Zepetnek et al., 1992). Hence the finding that contractile force in TA and MG muscles in the partially denervated hindlimbs of SOD1^{G93A} transgenic mice was the same as that in the hindlimbs supplied by intact spinal roots (*despite* there being no increase in MU contractile force) was the first indication that the partial denervation of the muscles by root avulsion had "saved" the remaining MUs and prevented their progressive decline in number with age. When the L5 spinal root was avulsed for example, there was a significant reduction of 40–70% in MU numbers in the hindlimb muscles at 40 days of age. The remaining MUs in the partially denervated hindlimb muscles of

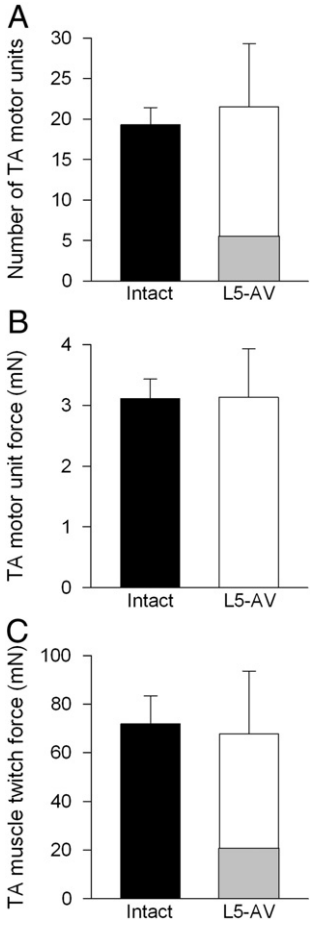

Fig. 8. Partial denervation of hindlimb muscles prevented the normal decline in the numbers of intact motor units in SOD1^{G93A} transgenic mouse. Histograms of the motor unit number + SE (A) motor unit twitch forces (B) and muscle twitch forces (C) in the tibialis anterior muscle of intact hindlimbs at 90 days of age compared with the predicted (gray bars) and the actual (white bar) number of motor units and the recorded TA motor unit and muscle twitch force after L5 spinal root avulsion (L5-AV).

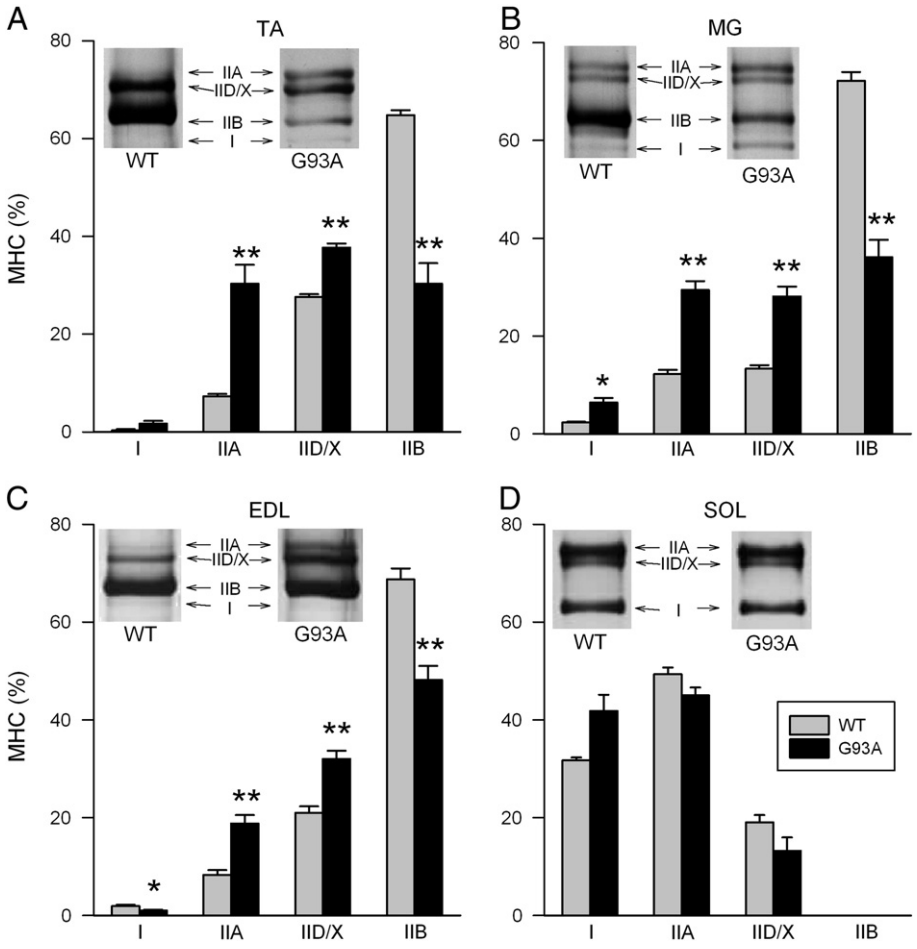

Fig. 9. Myosin heavy chain (MHC) isoforms in partially denervated TA (A), MG (B), EDL (C) and SOL (D) hindlimb muscles in SOD^{WT} and SOD^{G93A} transgenic mice at 90 days of age after avulsion of the L5 spinal root at 40 days of age. $^{**}p<0.01$ and $^{*}p<0.05$.

the $SOD1^{G93A}$ transgenic mice at the time of the avulsion all survived to 90 days of age. Importantly, the number of surviving functionally intact MUs in the partially denervated hindlimbs supplied by one of two spinal roots at 90 days of age was not significantly different from the number in the intact hindlimbs where the muscles were innervated by both spinal roots (Figs. 6 and 7). The considerable fiber type conversion from type IIB through to type IIA and I in the partially denervated muscles demonstrated that there was indeed, increased recruitment of the smaller motoneuron pool during asymptomatic disease that resulted in striking survival of functional MUs.

One cohesive explanation for the activity-induced fiber type conversion toward type IIA and I concurrent with sustained survival of MUs in the partially denervated hindlimbs of the $SOD1^{G93A}$ transgenic mouse is the evidence (1) for activity-dependent decline in axon size (Gordon et al., 1997) and (2) that more motoneurons survive in the $SOD1^{G93A}$ transgenic mouse after axotomy (Kong and Xu, 1999) when the caliber of the axons central to the site of nerve transection declines (Davis et al., 1978; Gordon, 1983; Gordon et al., 1991). Electrical stimulation of motoneurons and their muscle units for 12 h of each day promotes a type IIB to I transition in muscle fiber types and slow and less forceful muscle and MU contractions that do not fatigue. In addition, the activity induces a rapid decline in nerve conduction velocities and conversion of motoneuron electrical properties from fast to slow (Gordon et al., 1997; Munson et al., 1997; Gordon et al., 2004b). Conduction velocity is directly proportional to nerve fiber size such that the reduced conduction velocities reflect reduced size of the nerve fibers. It follows that the conversion from type IIB to IIA and I in the muscles in $SOD1^{G93A}$ transgenic mice is paralleled by changes in the motoneuron properties including reduced size of the motoneurons and their axons. Findings that axon diameters decline significantly after partial denervation (Havton et al., 2001) concur with the conclusion that the nerve fiber calibers decline in parallel with type IIB→type IIA and I muscle fiber conversion after partial denervation. Therefore, the preferential survival of the MUs with smaller axons during the lifespan of the $SOD1^{G93A}$ transgenic mouse and our new evidence for the survival of the more active MUs in partially denervated hindlimbs that convert progressively from type IIB to IIA and I, may be related, at least in part to their smaller axons. Moreover, the paradoxical finding that cutting the peripheral nerve (axotomy) in $SOD1^{G93A}$ transgenic mice actually increased the survival of ventral root axons is a second line of evidence that motoneurons with small axons survive better than larger axons in the $SOD1^{G93A}$ transgenic mouse (Kong and Xu, 1999). Consistent with these two lines of evidence, a plateau in numbers of functional MUs was reached at 100–110 days of age during the symptomatic disease after the rapid decline in MU numbers during the asymptomatic phase and progressive loss of the MUs containing the type IIB and IID/X muscle fibers (Hegedus et al., 2007). Yet reduced neurofilament content and smaller motor nerve fibers in the SOD^{G37R} transgenic mouse model of ALS where one allele of each neurofilament gene was disrupted did not alleviate motor axon loss (Nguyen et al., 2000). Alternative explanations including activity-dependent changes in gene expression in the neurons will have to be considered for the "saving" of functional motor units by partial denervation and/or axotomy. Motoneurons that supply type IIB and

IID/X muscle fibers have been referred to as vulnerable as opposed to those non-vulnerable motoneurons supplying type IIA and I fibers (Saxena et al., 2009). An exciting finding was that, in three transgenic mouse models, the vulnerable motoneurons upregulate endoplasmic reticulum (ER) stress markers prior to muscle fiber denervation while the non-vulnerable motoneurons do not. Perhaps the activity-dependent type IIB to type IIA and I by partial denervation in this study might reduce ER stress sufficiently to promote survival of functionally intact motor units. This possibility is imminently testable.

Conflicting data on the effects of imposed exercise programs on disease progression in mouse models and human ALS (Chung et al., 1992; Drory et al., 2001; Scarmeas et al., 2002; Krivickas, 2003; Chio et al., 2005; Kaspar et al., 2005) are likely explained by the different types of exercise and the effects that they mediate on muscle fiber composition. Daily amount and not pattern of activity determines muscle fiber composition: >5% daily activity converted muscle fibers from type IIB to type I, daily activity of <5% converted fibers to type IIA, and <0.5% daily activity favors type IIB fibers (Donselaar et al., 1987; Kernell et al., 1987; Kernell and Eerbeek, 1988; Gordon and Pattullo, 1993). Orderly MU recruitment from small slow fatigue resistant to the largest fatigable MUs favors isometric muscle contractions during postural movements with progressive recruitment and hence activation of the largest and fastest MUs during movements that overcome gravity and move limbs (Gordon and Pattullo, 1993; Gordon et al., 2004b). Consequently, our findings of survival of MUs with fatigue resistant muscle fibers in $SOD1^{G93A}$ transgenic mice indicate that daily low frequency activation for at least 5% of each day rather than exercises promoting brief high activation rates could benefit ALS patients by prolonging MU survival. The motor deficits and hastened disease onset in $SOD1^{G93A}$ transgenic mice that were exercised in short high intensity bursts (Mahoney et al., 2004) concur.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2009.10.021.

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