Amyotrophic lateral sclerosis - evidence of early denervation of fast-twitch muscles

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Abstract

In the SOD^{G93A} transgenic ALS mouse model, motoneuron death has not been reported earlier than 90 days of age, the so-called pre-symptomatic phase. We recorded contractile force from the fast-twitch medial gastrocnemius and slow-twitch soleus muscles and their constituent motor units to determine the number of intact motor units during the asymptomatic phase of disease. We found an early, selective and progressive loss of the largest and fastest motor units in the MG muscle. No such loss was evident in the slow-twitch soleus muscle. This data indicates selective axon die-back of the motoneurons that innervate the largest and fastest motor units. This die-back occurs prior to the death of the motoneurons in the spinal cord. Evidence of fiber-type conversions to more fatigue-resistant motor units suggested that this early loss may be slowed by increasing neuromuscular activity.

Keywords: Amyotrophic lateral sclerosis, motor units, muscle denervation, G93A transgenic mouse.

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The adult-onset disease of Amyotrophic lateral sclerosis (ALS) (also known as Lou Gehrig's disease and motor neuron disease) is a progressive, lethal, degenerative disorder of motoneurons. The disease is characterized by progressive weakness, paralysis, atrophy of skeletal muscle, and by premature death [5]. The hallmark of ALS is selective degeneration of motoneurons in the spinal cord, brainstem nuclei, and cerebral cortex. More than 100 mutations in the gene encoding the cytosolic anti-oxidant enzyme Cu/Zn superoxide dysmutase (SOD1) have been identified in the familial form of ALS (fALS) which comprises ~10% of all ALS cases [21]. Several of these mutations have been expressed in transgenic mouse models of fALS with the glycine to alanine conversion at the 93rd codon being the most common one. This G93A transgenic mouse, where the mutation is expressed in high copy number, demonstrates misfolding and abnormal aggregation of the SOD1 in the motoneurons, a feature that is becoming well recognized as a cardinal feature of the disease [3, 26]. Nonetheless, how and why the disease process is initiated are not yet fully understood [21].

The number of motoneurons in the spinal cord of the G93A transgenic mouse was reported to be normal at 80 days of age after which the number decreased during the symptomatic phase of the disease when muscles were reported to become progressively paralyzed until death at 5 to 6 months [4, 12]. Prior to 90 days of age, the disease was referred to as pre-symptomatic as there were no obvious visible features of the disease (see also [20]). Nonetheless, both anatomical and behavioral deficits have been reported during this pre-symptomatic phase. These include deficits in gait during treadmill locomotion [33] and signs of muscle denervation as early as 50 days [9, 22]. Electromyographic studies that aimed to determine the onset of disease in the G93A transgenic mice provided evidence for muscle denervation earlier than 90 days. However, these studies do not provide consistent data [2, 16, 27, 28, 29]. Very few human ALS studies included recordings of both electromyographic signals (EMG) and force [6, 19, 32]. In the mouse, only one laboratory to our knowledge has isolated skeletal muscles for recordings of muscle contractile forces [7]. In a study in which a retrograde tracer was injected into the medial gastrocnemius (MG) muscle in the same G93A mouse, normal numbers of motoneurons were reported between 49 and 56 days but there was a decrease to 36% of the normal number at 126 days of age when the overt symptoms of disease are severe [20].

In this study, we have systematically investigated the contractile force of muscles in the hindlimbs of the mSOD1 mouse during the pre-symptomatic phase of the

disease, i.e. ages less than 90 days. We developed a method of motor unit (MU) counting, the incremental twitch subtraction motor unit number estimation (ITS-MUNE) [18], in order to count the MUs in the mSOD1 mice and compare these numbers with those in mice that express normal human SOD1, referred to here as nSOD1 as well as wild type control mice. Our data demonstrate that functional MUs are lost during the pre-symptomatic phase of the disease *exclusively* in fast-twitch muscles. Findings that reduced axonal size after axotomy conferred protection for ALS motoneurons [17] and evidence of activity-dependent conversion of some of the surviving fast fatigable MUs with type IIB muscle fibers to more fatigue resistant units [14], suggest that induced neuromuscular activity has a protective effect on functional MUs in ALS.

Materials and Methods

Animals

Transgenic mice were obtained from Jackson Laboratories, USA to be used in this study. The mSOD1 transgenic mice expressed a high copy number of the human SOD gene with a glycine to alanine base pair mutation at the 93rd codon of the cytosolic Cu/Zn superoxide dismutase (SOD1) gene (B6JSL-TgN (mSOD1) or a high copy number of normal human SOD1 gene (nSOD1; B6JSL-TgN (SOD1) The transgenic male mSOD1 and nSOD1 mice were bred to non-transgenic B6JSL hybrid females, and the resulting progeny were identified using standard PCR protocol for the human SOD1 [24] performed on ear biopsy samples taken at the time of weaning (approximately 21 days of age). The mice were identified using ear punches, and kept in standard animal housing with free access to food and standard rodent chow.

The University of Alberta Health Sciences Laboratory Animal Ethics committee approved all experimental procedures, which were in accordance with the Canadian Council for Animal Care.

Surgical preparation and electrophysiological recordings in vivo

Under surgical anesthesia, the tendons of the fast-twitch medial gastrocnemius (MG), and the slow-twitch soleus (Sol) muscles in the hindlimbs of the mice were isolated at the ankle and tied with 4-0 silk thread for attachment to a strain gauge (Kulite model KH-102). Two silver wire electrodes were sutured alongside the sciatic nerve for stimulation and recording of evoked isometric muscle forces. The force was amplified and displayed on a monitor using Axoscope Software (Version 8-0, Axon instruments, USA). Muscle length was adjusted for maximum isometric twitch force in response to suprathreshold stimulation of sciatic nerve. Twitch and tetanic forces were recorded in response to single and repetitive supramaximal stimulation of the sciatic nerve at 0.5Hz and 100Hz.

To determine average force of the MUs in each of the 2 muscles, we progressively recruited all-or-none increments in twitch force by increasing the stimulus voltage incrementally. The sciatic nerve was stimulated with a range of pulse amplitudes from just threshold progressively through to about two-thirds maximal stimulation. A total of 150–300 compound muscle twitches were collected and rank ordered according to peak twitch force. Repeat stimuli were given at each pulse amplitude to record the change in isometric twitch force resulting from statistical alternation of motor axon excitation. We randomly selected 15 twitches and used the ITS-MUNE method of subtracting 2 rank-ordered whole muscle twitch force responses, in order to generate candidate individual MU twitch force responses. These responses were either rejected or accepted as representative single MU force responses [18]. Thereby, we determined MU twitch forces and calculated number of MUs by the ratio of the whole muscle and average MU twitch forces.

Statistics

Data are presented as means $\pm$ standard errors (SE). Statistical significance between experimental and control groups was assessed using a Students' t-test (SPSS, Version 8.0). Differences were considered statistical significant at $p<0.05$. Statistical significance at $p<0.01$ is shown in the figure as a double asterisk (**). Power calculations were completed to confirm sufficient power and/or sample size using PC-Size (STATOOLS, Version 2.13, 1986).

Results and Discussion

Muscle and motor units in fast- and slow-twitch muscles in the pre-symptomatic G93A transgenic mouse model of ALS

To resolve whether all functional MUs are intact prior to 90 days of age in the mSOD1 transgenic mouse model of ALS, we recorded muscle and MU forces in the slow-twitch Sol and the fast-twitch MG muscles in pre-symptomatic mSOD1 and nSOD1 transgenic mice and used the ITS-MUNE method to count the number of intact MUs. Prior EMG studies had indicated significant losses as early as 47 days in the MG muscle [8]. In the light of evidence that fast-twitch muscle fibers become denervated prior to 90 days [22], we investigated both slow- and fast-twitch muscles, the Sol and MG muscles, respectively.

We first made recordings of whole muscle force at 80 days of age when it had been reported that there was no loss of motoneurons in the spinal cord of the G93A mouse model of ALS [4, 12]. As shown in Fig. 1, the whole muscle twitch force of the fast-twitch MG muscle in the G93A mSOD1 mice was already significantly reduced in contrast to the force of the slow-twitch Sol muscle in the same mice that was not. When we recorded muscle force in the muscles earlier in life, we

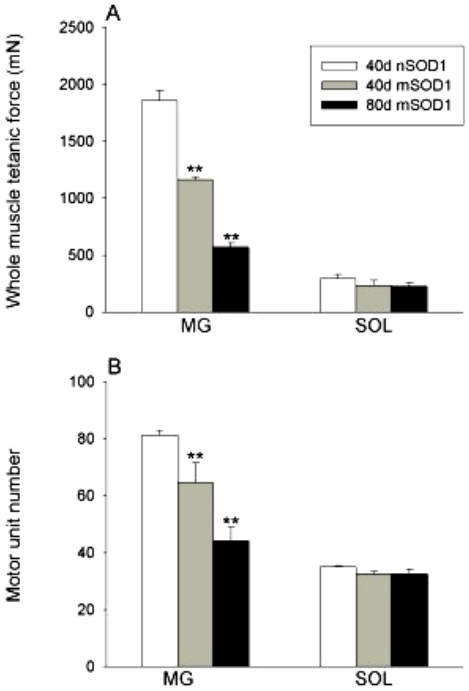

Figure 1: *Mean ± SE of the isometric tetanic forces (A) and the number of intact motor units (B) in the fast-twitch MG and the slow-twitch Sol muscles at 40 and 80 days of age in the G93A transgenic mouse model of ALS (mSOD1) as compared to the 40 day old mice that expressed normal human SOD1 (nSOD1).*
*** denotes statistical significance with p<0.01.*

found that there was already evidence of reduced muscle force in the MG muscle at 40 days of age, a reduction in muscle contractile force of ~35%. Calculation of the numbers of functionally intact MUs at 40 and 80 days of age revealed that the number of intact MUs was significantly reduced in the fast twitch MG muscle and that the number continued to decline with age during the pre-symptomatic phase of the disease (Figure 1B). The slow-twitch Sol muscle contrasted with the fast-twitch MG muscle in not displaying any loss in MU numbers during the asymptomatic phase of the disease and no significant decline in the contractile force.

These data demonstrate first, that the motoneurons which innervate the fast-twitch muscles are preferentially affected by ALS leading to reduction in the number of innervated MUs. Because no loss of motoneurons was detected in the spinal cord of G93A mSOD1 mouse prior to 80 days of age, these data provide supportive evidence that there is axonal die-back in ALS [15] with loss of motoneurons being detected only during the symptomatic phase of the disease after 90 days [4, 12]. The sensitivity of our method of counting functional motor units appeared to have been sufficient to detect a small but significant decline in intact MUs by 40 days of age. Backlabelling of motoneurons with fluorogold injected into the MG muscle between 49 and 56 days in the same G93A mouse was not sufficiently sensitive to detect the small decline at the early stages of pre-symptomatic disease [20].

The reduction in both muscle contractile force as well as the number of functional MUs in the fast-twitch muscle was surprising, particularly for the muscle at 40 days. Normally, partial loss of MUs from skeletal muscles is compensated for by enlargement of remaining MUs by axonal sprouting: axonal sprouts that emerge from the first node of Ranvier or from the terminals of intact motor units reinnervate denervated muscle fibers to enlarge MUs up to a maximum of ~5-fold [10, 23, 30, 31, 34]. This capacity for sprouting is very effective in compensating for loss of motoneurons to poliomyelitis and in partial nerve injuries [10]. The enlarged MUs may be sufficient to sustain normal levels of contractile force in the partially denervated muscles so long as more than 15-20% of intact MUs remain [10, 23, 34]. In the G93A transgenic mouse model of fALS on the other hand, even a reduction of 20% in the number of MUs in MG muscle at 40 days of age, was accompanied by the greater reduction of ~35% in the mean contractile force of the MG muscle.

The explanation for the greater reduction in muscle contractile force than MU number and the apparent inability of the remaining MUs in the fast-twitch MG muscle to undergo compensatory sprouting is to be found in the fiber type composition of this muscle and the reported inability of the MUs containing type IIB muscle fibers to sprout in the G93A mSOD1 transgenic mouse [9, 22]. The mouse MG muscle contains <10% of muscle fibers that express the slow myosin heavy chain (MHC type 1), most of the muscle fibers expressing the fast MHCs type IIX/D and IIB [13]. MU forces normally increase from the smallest MUs that comprise the type I muscle fibers to the progressively larger MUs containing type IIA, IIX/D and IIB muscle fibers; the largest force producing MUs are those that contain type IIB muscle fibers [11]. The dispro-portionate decline in whole muscle contractile force in the fast-twitch MG muscle as compared to the loss of the MUs (Fig. 1), is consistent with a preferential loss of the largest and fastest MUs that contain the type IIB and possibly the type IIX/D muscle fibers AND the failure of the remaining MUs to enlarge by sprouting. It follows that muscle contractile force should decline disproportionately with MU numbers until only type I and type IIA muscle fibers remain innervated. At that point in time, effective enlargement of the smaller MUs that contain type I and IIA muscle fibers may be observed. Indeed, such enlargement of MUs has been described during the symptomatic phase of the disease

after 90 days of age in fast-twitch as well as slow-twitch muscles in the G93A transgenic mouse model of ALS [25]. Slow-twitch Sol muscles, in contrast to the fast-twitch MG muscles, are comprised of type I and type IIA muscle fibers [1] and their motor nerves display effective sprouting when functional MUs are lost during the symptomatic phase of disease (Gordon and Tam, unpublished data).

Prior EMG studies had indicated significant losses in functional MUs as early as 47 days in the MG muscle of the G93A transgenic mouse model of ALS [8]. Our MU number estimations from force recordings provide data to confirm the conclusions from that study and to resolve some of the controversies concerning the time course and onset of MU loss that emerged from comparisons of different studies. Further, our findings are consistent with the evidence of the onset of muscle denervation during the asymptomatic phase of disease in the same transgenic mouse [22].

Concluding Remarks

There is a rapid attrition of the MUs in fast-twitch but not slow-twitch muscles from birth onwards in the phase of the disease in the mSOD1 transgenic mouse that was previously believed to be asymptomatic. The finding that the contractile force of the whole MG muscle declines relatively more than the number of the MUs is consistent with 1) a selective loss of the largest and most fatigable MUs and 2) the absence of effective sprouting in the remaining intact MUs to compensate for the loss of these MUs. The selective vulnerability of the largest and fastest MUs in ALS may explain the rapid onset of weakness in affected muscles in ALS patients.

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