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Length-dependent axo-terminal degeneration at the neuromuscular synapses of type II muscle in SOD1 mice

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Abstract

In motor neuron diseases, there is a prolonged period of time before any clinical symptoms begin to appear. During this time, distal axonal degeneration, or “dying back” axonopathy, begins to occur before the onset of clinical symptoms and motor neuron death. This preclinical degeneration is a hallmark of motor neuron diseases in both animal models and human patients. Generally, in muscles with mixed fiber types, distal degeneration occurs in fast-fatigable α -motor axons innervating type IIb muscle fibers before axons innervating slow, type I muscle fibers. We investigated whether the “dying back” axonopathy in a pure fast-fatigable α -motor axon nerve is a length dependent process. The lateral thoracic nerve (LTN) exclusively consists of motor nerves that innervate the very thin cutaneous maximus muscle (CMM) that solely contains type II neuromuscular synapses. We characterized the LTN and CMM synapses both morphologically and physiologically in the superoxide dismutase 1 (SOD1) mutant mouse model of amyotrophic lateral sclerosis (ALS). By 60 days of age, there was a significant “dying back” phenomenon at the caudal region while the rostral region remained intact. The longer axons innervating the caudal region appear to be more susceptible to degeneration in the SOD1 mouse indicating that the axonal degeneration of motor neurons innervating type II fibers is a length dependent process. Additionally, we identified how the simplicity of the LTN-CMM system offers a better method to investigate axon degeneration in an ALS mouse model and may be used to investigate possible therapeutic compounds for axon protection and regeneration.

INTRODUCTION

Motor neuron diseases are a debilitating group of neurodegenerative diseases that greatly impact the quality of life of patients. One such disease is amyotrophic lateral sclerosis (ALS), which is characterized by a progressive loss of motor neurons, eventually leading to

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muscle wasting and finally death (Cleveland and Rothstein, 2001). While there are both sporadic and familial forms of ALS, there is very little known about the actual causes of the disease. Many mutations have been discovered to be associated with familial forms of ALS. One mutation, the glycine 93 to alanine point mutation in the superoxide dismutase 1 gene (SOD1) (Rosen et al., 1993), has proven to be a useful manipulation for generating a popular mouse model of ALS by highly expressing the mutant SOD1 gene (Gurney et al., 1994, Wong et al., 1995). There have been many studies done on this mouse model to characterize the progression of the disease within the mouse. The earliest signs of disease begin to show up at around P45 and manifest as a significant decline in the number of innervated neuromuscular junctions (NMJs) (Azzouz et al., 1997, Frey et al., 2000, Fischer et al., 2004, Schaefer et al., 2005, Pun et al., 2006, Hegedus et al., 2007). This early denervation can also be detected as a decline in the amplitude of the compound muscle action potential (CMAP) using electrophysiological analysis (Azzouz et al., 1997, Hegedus et al., 2007). It is only later, around 90 days, that clinical symptoms begin to appear and degeneration becomes evident in the ventral roots and motor neuron cell bodies (Azzouz et al., 1997, Frey et al., 2000, Fischer et al., 2004, Schaefer et al., 2005, Pun et al., 2006, Hegedus et al., 2007).

The most widely used system to study the progression of neurodegeneration in the SOD1 mouse model is the sciatic nerve and the muscles it innervates. The sciatic nerve is approximately 1.5 to 2 cm in length and is composed of sensory, autonomic and motor fibers (Griffin et al., 2010). Studies have shown that type IIb fast-fatigable motor fibers have a selective vulnerability for degeneration in the SOD1 mouse compared with the type I or IIa subtypes (Frey et al., 2000, Pun et al., 2006, Saxena and Caroni, 2007, Saxena et al., 2009, Saxena et al., 2013). It is also well known that the largest motor neurons die first, pointing towards an inability to keep up with a high metabolic demand (Hegedus et al., 2007, Saxena et al., 2013). While the sciatic nerve consists of mixed axonal populations, there have not been any extensive studies done on very long nerves of a single nerve type within the SOD1 mouse. Previously, we had characterized the lateral thoracic nerve (LTN) and the cutaneous maximus muscle (CMM) system as a novel system for studying regeneration exclusively in very long motor nerves innervating type II fibers (Pan et al., 2012). The CMM is only 5–10 muscle fibers thick and spans the entire back of the mouse, allowing for up to 1000 NMJs to be examined (Theriault and Diamond, 1988a, b, Griffin et al., 2010, Pan et al., 2012). The LTN innervates the entire surface of the CMM, providing nerves 4–5 cm in length for study (Griffin et al., 2010). This is especially useful when characterizing the selective sensitivity for degeneration based on axon length as one can study different locations along the same nerve within the same muscle.

In order to accurately investigate the earliest signs of degeneration of the type II motor fibers, one must take into account the variations brought on by different genetic backgrounds. The B6SJL mixed background SOD1 mouse model, used in the studies mentioned earlier, present with overt clinical symptoms around P90 with a life span of around 130 days (Azzouz et al., 1997, Fischer et al., 2004, Heiman-Patterson et al., 2005, Hegedus et al., 2007, Hegedus et al., 2008). In contrast, mice with the C57BL/6J background have a delayed disease onset of around 110 days and an extended life span of approximately 150 days (Gurney et al., 1994, Chiu et al., 1995, Dobrowolny et al., 2005, Heiman-Patterson et al., 2005, Wooley et al., 2005). The mice on the B6 background show

an extended preclinical phase which provides a longer window to study the early stages of disease before the onset of clinical symptoms.

Despite the ongoing degeneration in SOD1 mice, there is some extent of axonal sprouting from the surviving axons in an attempt to reinnervate the vacated NMJs. This is evident by an observed increase in the size of the motor units and some sparse reinnervation seen morphologically in the SOD1 mice (Fischer et al., 2004, Gordon et al., 2004, Schaefer et al., 2005). While this partial reinnervation may be useful in the early phase of the disease, as the motor units increase in size, so does the metabolic demand. This eventually catches up with the surviving axons and they can no longer support the increased motor unit size which ultimately leads to the later phase of rapid decline (Azzouz et al., 1997). Another obstacle for regeneration is that the type II fast-fatigable fibers are fairly resistant to regeneration and do not sprout easily (Frey et al., 2000).

Here we used the C57BL/6J SOD1 mouse model to (1) examine whether the LTN-CMM system is an optimal system for studying temperospatial axonal degeneration, (2) investigate the length dependent degeneration of fast-fatigable, type II fiber, α -motor axons and (3) determine the extent of axo-terminal sprouting of type II fiber α -motor axons.

METHODS

Animals and operative procedure

All experiments and animal care procedures were conducted in accordance with the guidelines of the Johns Hopkins University Committee on the Use and Care of Animals. All breeding mice were obtained from Jackson Labs (Bar Harbor, Maine, USA). Mice associated with this study were housed in a facility at Johns Hopkins University.

Yellow fluorescent protein (YFP)-transgenic mice (B6.Cg-Tg(Thy1-YFP)16Jrs/J), in which all the motor axons express YFP fluorescence (Feng et al., 2000), were bred with SOD1 transgenic mice (B6.Cg-Tg(SOD1*G93A)1Gur/J)(Gurney et al., 1994), which express a G93A mutant form of human SOD1 and exhibit an ALS-like phenotype. Both lines used in this study were in the C57BL/6J genetic background. Progeny from breeding pairs were genotyped and grouped into SOD1-transgene carrying mice (SOD1) and wild type littermates (WT). During the 3 month course of the study, the SOD1 mice showed no signs of disease, no weight loss and no gait abnormality. Upon reaching either 30, 60 or 90 days of age, mice were anesthetized with isoflurane (Isosol, Vedco, St. Joseph, MO) and the hair was removed from the dorsal portion of the trunk of the body and legs with electric clippers. Mice then underwent electrophysiological analysis of the LTN and sciatic nerve function. Following electrophysiological recordings, mice were deeply anesthetized with 10% chloral hydrate (Sigma-Aldrich, St. Louis, MO) and the remaining hair was removed from the back of the body with an over the counter chemical depilatory. Each mouse was transcardially perfused with 1× PBS (Life Technologies, Carlsbad, CA) followed by perfusion with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 1× PBS. The skin, with the CMM attached, was removed from the back of the body and post-fixed in 2% paraformaldehyde for up to 16 hours and before being transferred to PBS for long term storage at 4 °C.

LTN-CMM electrophysiology

Mice were anesthetized using 2–3% isoflurane and the hair on their backs was removed by shaving with electric clippers. Compound muscle action potentials (CMAPs) were recorded from the LTN-CMM at 3 sites along the CMM using an Evidence 3102evo EMG system (Schreiber & Tholen Medizintechnik, Stade, Germany). In order to accurately determine each testing site, an easily identifiable baseline site between the second and third caudal vertebrae was established by palpating for the muscle mass on top of the third caudal vertebrae. Testing sites were marked 6, 24, and 36 mm anterior to the baseline site, with the recording and reference electrodes placed 6 mm on either side of the animal's midline. Two small incisions were made at the most caudal testing site and both the active and reference recording electrodes were placed just beneath the skin. A diagram of the experimental set up is shown in figure 3A. The grounding electrode was placed beneath the skin on the animal's side away from the recording and stimulating electrodes. An incision was made just below the left shoulder blade and the LTN was exposed proximal to where the nerve enters the CMM. Using a hooked electrode, the middle branch of LTN was isolated and stimulated (0.05 ms, 1 Hz). Stimulation was optimized with respect to both polarity (preferred polarity yielding the shortest CMAP latency) and intensity (supramax stimulation defined as 3 mA above the amount of current required for the size and shape of the CMAP to remain unchanged). CMAPs from the LTN-CMM system were then recorded and amplitudes and latencies were analyzed as measures of neuromuscular function. Repetitive stimulation schedules (optimized stimulation at 1, 5, and 10 Hz) were used to ensure stable electrode positioning. After testing was completed at the caudal site, the active and reference recording electrodes were cleaned and the electrophysiology procedure was repeated moving up towards the head at the medial and then the rostral testing sites.

Sciatic nerve electrophysiological experiments

A standard sciatic nerve electrophysiology protocol was followed using an Evidence 3102evo EMG system. Briefly, mice were anesthetized using 2–3% isoflurane and the left sciatic nerve was electrically stimulated by a very short electrical impulse (<0.2 ms). The proximal stimulation site was located at the sciatic notch and the distal stimulation site was located at the ankle. The recording electrode was placed in the plantar muscle of the foot pad and the CMAP was recorded. A diagram of the electrode placements is shown in figure 6A. The distance for the proximal stimulating site was approximately 31 mm and the distance for the distal stimulating site was approximately 12 mm from the recording electrodes. The amplitude of the CMAP in WT and SOD1 mice was compared at 30, 60, and 90 days of age.

Muscle type staining

Samples of the CMM from each recording site were collected immediately after recording, flash-frozen with liquid nitrogen and stored at –80 °C. Soleus muscles were obtained and prepared in a similar manner. Tissues were then immersed in TissueTek (Electron Microscopy Sciences) and cryosectioned at a thickness of 20 µm before both H&E and fiber type staining protocols were performed. Standard H&E staining protocol was followed (Thermo Fisher, Halethorpe, MD). For fiber typing, samples were permeabilized in a solution of 0.3% Triton X (Sigma-Aldrich) in 1× PBS, blocked with a Mouse on Mouse

blocking reagent (Vector Labs, Burlingame, CA), and then labeled with antibodies to identify type I myosin heavy chain (A4.840, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA) and type II myosin heavy chain (My32, Thermo Fisher).

LTN-CMM staining

Following perfusion, as described in animals and operative procedure, the skin from the back was removed and connective tissue covering the CMM was carefully dissected off of the whole back while leaving the skin intact. The back was then cut in half along the midline and one side was randomly selected for staining. The back half was permeabilized for 1.5 hours in a solution of 1% Triton X and 5% DMSO (Sigma-Aldrich) in 1× PBS and then stained with α -bungarotoxin conjugated to Alexa Fluor 594 (Life Technologies) to identify the postsynaptic NMJs. The back halves were then whole mounted onto slides using ProLong Gold Antifade (Life Technologies). CMM tissue from WT mice were also blocked in the same permeabilization solution with 5% goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). We blocked the tissue with Mouse on Mouse blocking reagent then stained the whole CMM from WT mice for synaptophysin (SV2 antibody from DSHB) and Schwann cells (S-100 antibody from Sigma-Aldrich) to identify endogenously expressed markers of the neuromuscular synapse.

LTN-CMM imaging

We imaged whole-mounted back halves using an Axio Imager fluorescence microscope with an Apotome apparatus (Zeiss, Jena, Germany) and a laser scanning confocal microscope (LSM 510, Zeiss). The backs were imaged at three different locations along the CMM corresponding to the caudal, medial and rostral regions used for the electrophysiological experiments (6, 24, and 36 mm from the baseline respectively). Z-stacks were taken for each image and were flattened with the ZEN software (Zeiss). The gamma and brightness was raised on the images in order to visualize axons with low YFP expression. Approximately 150 NMJs were imaged at each site per mouse.

Innervation analysis

To determine the extent of innervation, NMJs were counted and scored as either completely innervated or fully denervated. Complete innervation was counted as a total overlap of the pre-synaptic and post-synaptic staining comparable to control mice. Fully denervated NMJs were counted as no overlapping of the pre-synaptic and post-synaptic staining. The number of innervated NMJs was then divided by the total number of NMJs to determine the percentage of innervation. This percentage was then compared at the caudal, medial, and rostral regions between WT and SOD1 mice.

Axonal sprouting characterization

Axonal sprouting was analyzed using the same preparations from the innervation analysis. We scored thin, extra-terminal YFP-positive axons as sprouting axons and compared the presence of sprouts between WT and SOD1 mice. To calculate the percentage of terminal axonal sprouts we counted the number of extra-terminal sprouts and divided by the total number of NMJs.

Statistical analysis

All statistical analyses were performed with two-tailed Student's t test. Data that failed the normality test was analyzed using the Mann-Whitney Rank Sum Test. SigmaPlot software (Systat Software Inc., San Jose, CA) was used to perform the analyses and any value of $p < 0.05$ was scored as statistically significant. Graphed data are presented as mean $\pm$ SEM.

RESULTS

The LTN-CMM system is a thin muscle exclusively comprised of type II muscle fibers

Since fast-fatiguing α -motor axons innervating type II muscle fibers are very susceptible for degeneration in the SOD1 ALS mouse model (Frey et al., 2000, Pun et al., 2006, Saxena et al., 2009, Saxena et al., 2013), we characterized a system to exclusively study a large type II muscle and its NMJs. To do this, we first confirmed that the LTN-CMM system in the mouse only contained type II muscle. We collected the CMM from the body by removing the skin off the back of the trunk with the CMM remaining attached to the skin. After staining with hematoxylin and eosin, the CMM was identified as a thin layer of muscle closely associated to the dermis of the skin (figure 1A, black bar). We stained the CMM and soleus muscle for myosin heavy chain type I (A4.840) and type II (My32) to identify muscle fiber types. In the CMM, only type II fibers were present, as evidenced by the uniform staining in the muscle fibers of My32 (figure 1B and C). On the other hand, when the soleus muscle was simultaneously stained for type I and type II fiber markers, both were present (figure 1D). Since it is well known that the soleus muscle contains both fibers, this indicated that our staining protocol was reliable. Thus, we confirmed that the CMM only contains type II muscle fibers and can confidently be used to specifically study the degenerative properties of type II fibers in the SOD1 mouse model of ALS.

Whole-mounted LTN-CMM can be stained with Antibodies

One technical problem with using whole muscle tissue to investigate neurodegeneration is that many antibodies cannot penetrate deep into the intact tissue, thus requiring sectioning in order to get complete staining. While sectioning allows for easier staining, there is a trade off as it becomes more tedious afterwards to reconstruct complete NMJs to determine the extent of degeneration. We stained the CMM with SV2 and S-100 antibodies to visualize the neuronal and glial components of the neuromuscular synapses in whole-mounted tissue. We were able to achieve complete staining with the SV2 antibody as indicated by a complete overlap of the SV2 and α -bungarotoxin (α -btx) staining in the intact CMM of WT mice (figure 2 A–D). We also observed extensive Schwann cell labeling with the S-100 antibody (figure 2 E–H). The ability to carry out effective staining in whole tissue provides the LTN-CMM system the unique ability to observe all the components of the neuromuscular synapse in a single preparation.

Degeneration appears first in the longest axons of the LTN

To determine the earliest time point that axon degeneration can be detected in the LTN-CMM system of SOD1 mice, we analyzed them at 30, 60 and 90 days of age. Morphological changes at the NMJs were determined by the extent of denervation of the NMJs by scoring

the percentage of presynaptic YFP staining overlapping with the postsynaptic α -btX staining (figure 3B–D). To determine any physiological changes we measured electrophysiological recordings from 3 different locations on the back (figure 3A). We started with the most caudal recording site and moved rostrally, in order to preserve the shorter axons for later recordings at each site.

Neither axon nor terminal degeneration was detected at 30 days of age—At the earliest time point we investigated, there was no detectable difference between wild type mice and the SOD1 mice, both morphologically and physiologically. When we scored the percent of innervated NMJs at the caudal, medial, and rostral regions of the CMM (figure 3E), the WT mice had on average 99.9%, 99.4% and 99% innervation respectively (n=3). The SOD1 mice also had no degeneration with 97.1%, 99.4% and 99.7% innervation at the caudal, medial, and rostral regions respectively (n=4). The values in the SOD1 mice were comparable to those seen in the WT indicating that morphological changes have not occurred by P30 ($p>0.05$). We observed a similar result with the electrophysiological recordings taken at the 3 sites (figure 3F). In the WT mice, CMAP amplitudes on average were 0.27mA at the caudal region, 0.77mA at the medial region, and 2.02mA at the rostral region (n=15). The SOD1 mice had similar amplitudes ($p>0.05$) with the caudal region at 0.34mA, the medial region at 0.60mA and the rostral region at 1.90mA (n=17). The latencies at each region were also similar between the WT and SOD1 mice (data not shown).

Marked degeneration appears at the caudal region by 60 and 90 days—When we examined 60 day old mice, we observed evidence of degeneration in the SOD1 mice compared with the WT mice. The largest difference in NMJ innervation was seen at the caudal site with an average of only 44.5% of the NMJs being innervated in the SOD1 mice compared with 97.9% innervation in the WT mice (figure 4A–C; SOD1 n=3; WT n=4; $p=0.0093$). There was also a slight decrease in the number of innervated NMJs at the medial region of the back with 77.8% innervation in the SOD1 mice compared with 98.7% in the WT mice ($p>0.05$). At the rostral site, there was no significant difference between the WT and the SOD1 mice (97.1% and 95.8% respectively; $p>0.05$). Examining the neurophysiological changes in the SOD1 mice at 60 days revealed a decrease in the amplitudes of the CMAP at both the caudal (figure 4D; WT amplitude=0.36mA, SOD1 amplitude=0.16mA; WT n=21, SOD1 n=17; $p=0.00031$) and medial sites (figure 4D; WT amplitude=0.79mA, SOD1 amplitude=0.50mA; $p=0.029$). There was no significant difference in the amplitudes at the rostral site, in agreement with the lack of denervation seen at the rostral region (figure 4D; WT amplitude=1.39mA, SOD1 amplitude=1.63; $p>0.05$).

At 90 days of age, we observed further degeneration of the LTN axons. The caudal site of the SOD1 mice showed an even greater decrease in innervation with only 24.3% innervated compared to 88.4% in the WT (figure 5A–B, E; SOD1 n=4, WT n=2; $p=0.00091$). The medial region of the SOD1 mice was only 55.3% innervated while the WT mice had 90.7% innervation (figure 5E; $p=0.0050$). It was only at 90 days that degeneration in the SOD1 mice was apparent at the rostral site (figure 5C–D, E). Only 59.8% of the NMJs were innervated in the SOD1 mice while the WT mice had 93.9% innervation (figure 5E;

$p=0.029$). When we compared the CMAP amplitudes of WT and SOD1 mice, we observed a further decrease in the amplitudes at the caudal site with the SOD1 mice at 0.13mA compared to the WT amplitude of 0.27mA (figure 5F; WT $n=19$, SOD1 $n=19$; $p=0.013$). The medial site also continued to decline with the SOD1 amplitude at 0.37mA while the WT remained normal at 0.75mA (figure 5F; $p=0.020$). The amplitude at the rostral site in the SOD1 mice was 1.66mA which was slightly below the WT amplitude at 2.13mA (figure 5F; $p>0.05$).

Physiological changes at the distal LTN occurs earlier than the sciatic nerve

We next compared the time course for which we observed physiological changes in the LTN-CMM system with the time course in the sciatic nerve of SOD1 mice. Similarly to the LTN-CMM, the sciatic nerve did not show any difference in the CMAP amplitude between the WT ($n=6$) and SOD1 ($n=6$) mice at 30 days of age at both the proximal and distal sites (figure 6B; $p>0.05$). When we examined P60 mice, there was again no significant difference in amplitude between the WT ($n=7$) and SOD1 ($n=8$) mice at both sites (figure 6C; $p>0.05$). This differs from what we observed in the LTN-CMM system as there was a significant decrease in the CMAP amplitude at the caudal site by 60 days, whereas in the sciatic nerve there was no decrease in the amplitudes at either recording site. Only at 90 days were we able to detect a significant decrease in the CMAP amplitudes in the sciatic nerve at both the distal site ($p=0.050$) and proximal site ($p=0.003$) (figure 6D; WT $n=18$, SOD1 $n=19$). The ability to detect degeneration earlier in the LTN-CMM system makes it a more robust and attractive model to study the earliest time point of degeneration in the SOD1 mice.

Limited sprouting of the surviving LTN axons

Next, we searched for evidence of axonal sprouting from the surviving axons in the SOD1 mice. In order to do this, we used the same preparations as those used to characterize the extent of NMJ denervation and looked for the presence of extremely thin terminal sprouts. Terminal sprouting was not robust in the 30 day old mice, as was expected given the lack of degeneration seen in figure 3. We were able to detect some level of sprouting at the caudal and medial sites in both the 60 and 90 day old SOD1 mice (figure 7). Figure 7 A–C shows an image of two terminal sprouts in a 60 day old SOD1 mouse as indicated by the white arrows in figure 7C. While there is evidence of sprouting, there is a very limited capacity at 60 days for sprouting as evidenced by our quantification (figure 7D, red bars, $n=5$). At the caudal region only 0.35% of NMJs had a terminal sprout and the medial region had 0.42%. There were no sprouts detected at the rostral region as there was no degeneration observed to initiate the sprouting machinery. At 90 days, when the degeneration was more extensive, there was an increase in the percentage of NMJs with terminal sprouts (figure 7D, blue bars, $n=6$). The caudal, medial, and rostral regions had 1.47%, 1.0%, and 0.54% of NMJs with terminal sprouts respectively. Despite this increase, the extent of sprouting is still very limited and cannot keep up with the degenerative process. The presence of these sprouts, however limited, does indicate that type II fibers do have the capacity for some limited regeneration in the SOD1 mouse model. With the advantage of being able to whole mount the intact CMM, we were able to image the origin of the sprouts and easily determine whether it was a terminal sprout or not. The LTN-CMM system provides a unique model to

selectively investigate potential strategies for axonal protection and enhancing regeneration of type II motor fibers in SOD1 mouse models.

DISCUSSION

In this paper we utilized a novel model to investigate the length dependent degeneration of type II motor fibers in the SOD1 mouse model of ALS. By using the LTN-CMM system, it allowed us to observe that the longer axons in the caudal region showed more extensive degeneration at 60 days of age while the shorter rostral region only had moderate degeneration at 90 days. This degeneration was detected by both the extent of NMJs denervation as well as a functional decline using electrophysiological recordings of the CMAP.

Previous studies have shown that each muscle fiber type is affected differently, with type IIb being the most susceptible to denervation in the SOD1 mouse (Frey et al., 2000, Pun et al., 2006, Saxena and Caroni, 2007, Saxena et al., 2013). The varying time points of degeneration in the different types of motor fibers confounds the investigation of the effect of axon length on degeneration as various regions have unique motor fiber type makeups. If one region consists of more type IIb fibers, then it may show greater degeneration compared to a longer region consisting of more type I fibers. The LTN-CMM system eliminates any possible confusion with the different degeneration profiles of different motor fiber types as it only consists of type II fibers, which we verified in this study. This eliminates the variable of different fiber types and allows for a more consistent study of how the length of the axon affects its susceptibility to degeneration. Based on our findings, the degeneration of fast fatiguing, α -motor axons innervating type II muscle fibers is a length dependent process in the SOD1 mouse model.

Fischer et al (2004) showed that the degeneration in the SOD1 mouse model begins in the periphery before it is observed at the level of the spinal cord. While this study was instrumental in understanding that ALS is a peripheral neuropathy, it did not study in depth how the length of the peripheral axons affects their susceptibility for degeneration. They sectioned the gastrocnemius, soleus and tibialis muscles to determine the extent of degeneration. Each of these muscles are innervated by nerves of similar length in the mouse. Our study fills in this gap by providing evidence that the longer the axon, the earlier it degenerates. The ability to study length dependent degeneration in peripheral axons is easier to perform in the LTN-CMM system as axons of different length can be studied within the same muscle preparation. This is much easier than having to look at different muscles to generate a large difference in axon length. Using the CMM also allows for the entire muscle to be stained and whole mounted. We were also able to record electrophysiological data from 3 different lengths within the same muscle to generate more information about how there is a length dependent functional decline. By being able to collect all of this information from a single muscle, the LTN-CMM system is a much simpler system to study length dependent degeneration.

In order to study how axon length affects degeneration susceptibility at the earliest stages of disease, we used SOD1^{G93A} mice on the C57BL/6J background. The C57BL/6J SOD1 mice

show clinical symptoms on average around 110 days and die around 150 days (Gurney et al., 1994, Chiu et al., 1995, Dobrowolny et al., 2005, Heiman-Patterson et al., 2005, Wooley et al., 2005), while those on a mixed background show clinical symptoms starting at around 90 days and die by 130 days (Azzouz et al., 1997, Fischer et al., 2004, Heiman-Patterson et al., 2005, Hegedus et al., 2007, Hegedus et al., 2008). The BL/6J background is advantageous in studying earlier degeneration because the later onset of clinical symptoms allows for more time to study the time course of degeneration in these long axons. We observed that there is extensive degeneration almost 2 months prior to the onset of clinical symptoms in the BL/6J SOD1 mice. The early time point of the LTN degeneration is advantageous since it allows for a quicker investigation of degeneration of the type II motor synapses well before the onset of symptoms. Also, using the LTN-CMM model in the C57BL/6J backgrounds gives a few more weeks of studying degeneration before the onset of clinical symptoms and may be a better system to study early intervention therapies.

In SOD1 mice, it has been shown that there is a limited amount of intrinsic sprouting in the early stages of the disease. As the disease progresses, this capacity for sprouting gets overwhelmed and cannot keep up with the rate of degeneration (Gordon et al., 2004). The fast-fatigable type IIb motor fibers typically do not sprout easily (Frey et al., 2000, Pun et al., 2006). Due to the LTN-CMM system being made up of only type II motor fibers, we examined the extent of sprouting in the SOD1 mouse in order to determine whether this limited sprouting could be detected. There was some evidence of a few sprouts in some of the SOD1 mice, although most of these sprouts did not seem to be directed towards areas of denervation in particular. Ways to increase this limited capacity for sprouting may be a possible early therapy for ALS, and motor neuron diseases in general, as sprouting may help to maintain some of the motor function in the patient. Hopefully, tapping into the intrinsic capacity of these motor neurons for sprouting can help alleviate some of the devastating symptoms of motor neuron diseases and improve the quality of life for patients.

This study provided evidence that type II motor fibers degenerate in a length dependent process in the SOD1 mouse model. We believe that using the LTN-CMM system to study motor neuron degeneration is an elegant way to look at all the pieces required to determine the mechanism behind degeneration and to further study the regeneration of the surviving nerves.

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Highlights

1. Susceptibility of synapses of type II muscle to length dependent degeneration
2. Physiological and morphological dysfunction and degeneration of NMJs
3. Early signs of degenerative insults in the SOD1 mice.
4. Novel use of LTN-CMM to study pure motor nerve degeneration in the SOD1 mice

Figure 1.

The CMM is exclusively comprised of type II muscle fibers. (A) Musculoskeletal morphology shown through hematoxylin and eosin staining where the CMM is indicated by a black bar. (B–D) Myosin heavy chain immunostaining of the CMM where type I fibers were labeled with A4.840 (red) and type II fibers were labeled with My32 (green). (B) Low magnification fiber type immunostaining shows the CMM is comprised of only type II fibers. (C) High magnification of B. (D) Control with soleus tissue showing a mix of type I and type II fibers.

Figure 2.

Reliable antibody staining of the neuromuscular synapse components in the whole, intact CMM. (A–D) SV2 antibody staining. (A) SV2 staining of the pre-synaptic component of the neuromuscular synapse. (B) α-bungarotoxin staining of the post-synaptic component. (C) YFP expressing motor axons. (D) Overlay of A–C showing that all three stains overlap completely and all the terminals have SV2 staining. (E–H) S-100 antibody staining. (E) S-100 staining of Schwann cells. (F) α-bungarotoxin staining of the NMJs. (G) YFP expressing motor axons. (H) Overlay of E–G showing that the S-100 staining overlaps both the axons and the NMJs. Both axonal and terminal Schwann cells can be reliably stained.

Figure 3.

No evidence of axonal degeneration in 30 day old SOD1 mice. (A) Schematic diagram of our electrophysiology setup. LTN stimulation is near the shoulder (green) while the active (red) and the reference (black) recording electrodes are placed 6 mm on either side of the midline (dashed gray line). The three data collection sites are indicated by roman numerals with the caudal site measured 6 mm (I), the medial site measured 24 mm (II), and the rostral site measured 36 mm (III) anterior to the baseline (blue line). Representative CMAP recordings are shown below with their corresponding site denoted by the roman numerals.

(B–D) Example of fully innervated NMJs in a 30 days old WT mouse. (B) YFP positively labeled motor axons and terminals from YFP transgenic mouse. (C) α -bungarotoxin labeled NMJs. (D) Merged image of B and C showing all NMJs are innervated by the complete overlap of YFP and α -bungarotoxin staining. (E) There was no significant difference in the percentage of innervated NMJs at each of the collection site for 30 day old SOD1 (red, n=4) and WT (blue, n=3) mice (bars represent mean $\pm$ standard error). Numbers in parenthesis indicate total number of NMJs counted. (F) CMAP amplitudes at each collection site were not significantly different for 30 day old SOD1 (n=17) and WT (n=15) mice (points represent mean $\pm$ standard error).

Figure 4.

Axonal degeneration is evident by 60 days in SOD1 mice at the caudal site but not the rostral site. (A–B) A morphological comparison of the caudal site from 60 day old WT mice (A) and 60 day old SOD1 mice (B) showing far more denervated NMJs in the SOD1 mice than the WT. YFP expressing axons are shown in green and α -bungarotoxin labeled NMJs are in red. (C) A quantification of the percentage of innervated NMJs between 60 day old WT mice (blue, n=4) and SOD1 mice (red, n=3). The caudal region has the greatest decline in innervation while the rostral region remains relatively intact. Bars represent mean $\pm$ standard error. Numbers in parenthesis indicate total number of NMJs counted. (D) CMAP amplitudes at the caudal and medial regions in SOD1 mice (n=17) showed a significant decline in amplitude when compared to WT mice (n=21). Points represent mean $\pm$ standard error. * indicates $p < 0.05$.

Figure 5.

Axonal degeneration is apparent at the rostral site at 90 days of age in SOD1 mice. (A–B) Representative images at the caudal site of WT mice (A) and SOD1 mice (B) show increased degeneration with extensive denervation in the SOD1 mice while the WT remain fully innervated. YFP expressing axons are shown in green and α -bungarotoxin labeled NMJs are in red. (C–D) Representative images at the rostral site of WT mice (C) and SOD1 mice (D) show degeneration at the rostral site in SOD1 mice while the WT is intact. (E) Quantification of the percentage of innervated NMJs in 90 day old mice showing increased denervation at the caudal region and medial sites and significant degeneration appearing at the rostral region in SOD1 mice (red, n=4) compared with WT mice (blue, n=4). Bars represent mean $\pm$ standard error. Numbers in parenthesis indicate total number of NMJs counted. (F) CMAP amplitudes are significantly reduced at the caudal and medial regions in SOD1 mice (n=19) at 90 days when compared with WT mice (n=19). The rostral region also

shows a slight decrease in SOD1 mice but it did not reach significance. Points represent mean $\pm$ standard error. * indicates $p < 0.05$.

Figure 6.

The sciatic nerve shows a decrease in CMAP amplitude at 90 days. (A) Schematic diagram showing electrode placements for the left sciatic nerve recordings. The recording electrodes were placed in the foot pad as indicated by the black electrode. The sciatic nerve was stimulated separately at each of the green electrodes with the proximal or distal stimulation sites labeled. The proximal site was located 31 mm from the recording electrodes and the distal site was 12 mm from the recording electrodes. (B) At 30 days old, SOD1 mice (red, $n=6$) show no significant difference in recordings for both the distal and proximal stimulating sites when compared with WT mice (blue, $n=6$). (C) Again, at 60 days of age, SOD1 mice (red, $n=8$) do not show any significant difference from the WT mice (blue, $n=7$). (D) Only at 90 days of age do SOD1 mice show a significant decline in the CMAP amplitudes when stimulated at either the distal or proximal sites. The inset shows representative traces at the proximal site in WT mice (blue, $n=18$) and SOD1 mice (red, $n=19$) at 90 days old. Bars represent mean $\pm$ standard error. * indicates $p < 0.05$.

Figure 7.

SOD1 mice show very limited axonal sprouting. (A–C) Representative images of sprouting in a 60 day old SOD1 mouse (A) YFP labeled motor axons and presynaptic terminals. (B) α -bungarotoxin labeled NMJs. (C) Combined images of A and B showing that the medial region in an SOD1 mouse has the capacity for axonal sprouting with terminal sprouts indicated by white arrows. (D) Quantification of the percentage of NMJs with terminal sprouts at the three regions (caudal, medial, and rostral). 60 days old mice are shown in red (n=5). 90 days old mice are shown in blue (n=6). Numbers in parenthesis indicate the number of NMJs counted. Bars represent mean $\pm$ standard error.