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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.

Key words: motor neuron disease, neuromuscular, degeneration, innervation, motor axon, sprouting.

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 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 1.5–2 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, 2013). It is also well known that the largest motor neurons die first, pointing toward 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

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Abbreviations: ALS, amyotrophic lateral sclerosis; CMAP, compound muscle action potential; CMM, cutaneous maximus muscle; LTN, lateral thoracic nerve; NMJs, neuromuscular junctions; PBS, Phosphate-buffered saline; SOD1, superoxide dismutase 1; WT, wild type; YEP, Yellow fluorescent protein.

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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, 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.

EXPERIMENTAL PROCEDURES

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, USA) 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, USA) 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× Phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA) followed by perfusion with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) 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 h 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. CMAPs were recorded from the LTN–CMM at three 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 Fig. 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