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Altered terminal Schwann cell morphology precedes denervation in SOD1 mice

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ABSTRACT

In mice that express SOD1 mutations found in human motor neuron disease, degeneration begins in the periphery for reasons that remain unknown. At the neuromuscular junction (NMJ), terminal Schwann cells (TSCs) have an intimate relationship with motor terminals and are believed to help maintain the integrity of the motor terminal. Recent evidence indicates that TSCs in some SOD1 mice exhibit abnormal functional properties, but other aspects of possible TSC involvement remain unknown. In this study, an analysis of TSC morphology and number was performed in relation to NMJ innervation status in mice which express the G93A SOD1 mutation. At P30, all NMJs of the fast medial gastrocnemius (MG) muscle were fully innervated by a single motor axon but 50% of NMJs lacked TSC cell bodies and were instead covered by the processes of Schwann cells with cell bodies located on the preterminal axons. NMJs in P30 slow soleus muscles were also fully innervated by single motor axons and only 5% of NMJs lacked a TSC cell body. At P60, about 25% of MG NMJs were denervated and lacked labeling for TSCs while about 60% of innervated NMJs lacked TSC cell bodies. In contrast, 96% of P60 soleus NMJs were innervated while 9% of innervated NMJs lacked TSC cell bodies. The pattern of TSC abnormalities found at P30 thus correlates with the pattern of denervation found at P60. Evidence from mice that express the G85R SOD1 mutation indicate that TSC abnormalities are not unique for mice that express G93A SOD1 mutations. These results add to an emerging understanding that TSCs may play a role in motor terminal degeneration and denervation in animal models of motor neuron disease.

1. Introduction

In several animal models of motor neuron disease, degeneration begins in the periphery (Sagot et al., 1995; Balice-Gordon et al., 2000; Fischer et al., 2004). Evidence for a dissociation between motor neuron cell death and peripheral degeneration has been obtained from mice which overexpress mutations of the superoxide dismutase 1 (SOD1) protein which are found in cases of inherited ALS (Gurney et al., 1994). In these mice, inhibition of motor neuron cell death does not prevent development of muscle denervation (Gould et al., 2006). In other studies of SOD1 mice, intramuscular axonal arbors of living motor neurons have been imaged which do not innervate muscle fibers (Schaefer et al., 2005). These observations show that loss of motor unit function and weakness do not develop as a result of motor neuron cell death but rather as a result of peripheral degeneration.

The mechanisms which underlie this initial peripheral involvement are unknown. One possibility that has been considered is that normal interactions between muscle and motor neurons are disrupted. There is mixed evidence for a role of muscle-specific expression of mutant protein in determining disease phenotype in SOD1 mice with some studies suggesting a toxic influence (Sugiura et al., 2004; Wong and Martin, 2010) while others report that SOD1-expressing muscle exerts no influence on disease (Miller et al., 2006). Evidence obtained using whole muscle, syngeneic grafts to create surgical chimeras does not support a role for SOD1 muscle. We found that wildtype (WT) muscle did not inhibit SOD1 motor terminal degeneration when grafted into mice that express the G93A SOD1 mutation ($SOD1^{G93A}$) and that $SOD1^{G93A}$ muscle did not cause WT motor terminal degeneration when grafted into WT mice (Carrasco et al., 2010).

During reinnervation of grafted muscles, host Schwann cells (SCs) were found to accompany regenerating axons (Carrasco et al., 2010). Thus, a possible explanation for the appearance of motor terminal degeneration when SOD1 motor axons reinnervate WT or SOD1-expressing muscle is that interactions between SCs and motor axons, both of which express mutant protein, are sufficient to cause degeneration. Consistent with this is evidence that SC-specific expression of mutated SOD1 protein (G93A) causes no pathological effects (Turner et al., 2010), Other studies have produced inconsistent evidence for a role of mutant-expressing SCs. Using the same cre-mediated strategy (P_0 cre, (Feltri et al., 1999)), separate groups found that SC-specific partial reduction of mutant SOD1

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expression accelerates (Lobsiger et al., 2009) or modestly slows (Wang et al., 2012) disease progression in SOD1 mice that express the G37R or G85R mutation, respectively. The opposing results were attributed to different dismutase activities of the G37R and G85R SOD1 mutant proteins.

More recent evidence indicates that terminal Schwann cells (TSCs, or perisynaptic Schwann cells) in some SOD1 mice exhibit altered signaling linked to motor terminal action potential activity (Arbour et al., 2015). Such evidence is potentially significant because TSCs have an important but poorly understood trophic relation with motor terminals (Koirala et al., 2003; Reddy et al., 2003). No other information is available about TSCs in SOD1 mice. We therefore examined SOD1 TSCs at several ages using immunolabeling methods. The results show that TSC abnormalities are present before denervation commences in a pattern that correlates with future disease-related muscle denervation.

2. Methods

2.1. Animals

Three different transgenic mouse strains of either gender were used, each on a congenic C57BL6/J background to avoid genetic modifier effects of background strain on disease expression (Heiman-Patterson et al., 2011). Most work was conducted using mice which overexpress a transgene that carries the G93A mutation of human SOD1 protein (Gurney et al., 1994). Hereafter, these mice (B6.Cg-Tg(SOD1-G93A)1Gur/J) are referred to as B6.SOD1 mice. These mice exhibit changes in motor performance at about 50 days which are detectable by gait analysis but survive about 1 month longer (50% survival ~160 days) than the original mice carrying the same transgene on a mixed B6SJL background (Wooley et al., 2005; Heiman-Patterson et al., 2011). In some experiments, mice were used that express the human transgene for the G85R SOD1 mutation (B6.Cg-Tg(SOD1*G85R)148Dwc/J, hereafter $SOD1^{G85R}$). These mice express mutant SOD1 protein at much lower levels than $SOD1^{G93A}$ mice, and have a later disease onset (8–10 months) followed by uniformly rapid disease progression (Bruijn et al., 1997). Aged-matched C57BL6/J (hereafter WT) mice were used as controls. All mice were bred at Emory University or acquired from The Jackson Laboratory (www.jax.org, Bar Harbor, ME). All experiments were carried out in accordance with the Institutional Animal Care and Use Committees of Emory University.

2.2. Immunolabeling

Under general anesthesia, the medial gastrocnemius (MG) and soleus muscles were recovered and prepared for immunolabeling. Muscles were placed into 4% paraformaldehyde for 1 h. Muscles were washed in a 0.1 M phosphate buffered solution (PBS) and incubated at 4 C overnight in PBS containing 20% sucrose for cryoprotection. Sections (50 μm thickness) were obtained using a Cyrostat (Leica). Motor endplate acetylcholine receptors (AChRs) were labeled with Alexa 488 conjugated α-bungarotoxin (Molecular Probes). Axons and motor terminals (synaptic vesicles) were labeled with a mouse monoclonal antibody against the phosphorylated heavy fragment of neurofilament protein (SMI31, 1:400, Sternberger Monoclonal) and synaptic vesicles (SV2, 1:20, Developmental Studies Hybridoma Bank). Labeling was visualized using Cy5-conjugated secondary antibody (1:100, Jackson Immunoresearch Laboratories). Schwann cells (SCs) were labeled with a rabbit polyclonal antibody against S100B Ca^{2+}-binding protein (S100, 1:100, Dako) and visualized using a rhodamine-conjugated secondary antibody (1:100, Jackson Immunoresearch Laboratories). In addition to S100 labeling, SCs in some sections were also labeled with a goat polyclonal antibody against the low affinity nerve growth factor receptor p75 (NGFR p75, 1:50, Santa Cruz SC-6188) and visualized using a Cy5-conjugated secondary antibody (1:100, Jackson Immunoresearch Laboratories). DAPI staining was used to identify SC or TSC cell body nuclei. This staining was performed as per manufacturer's instructions using slide mounting media (Vectashield) which included DAPI.

2.3. Imaging

Z-axis stacks of images of neuromuscular junctions (NMJs) were obtained at sequential focal planes (1.0 μm separation) using an upright microscope equipped with a motorized stage (Leica). Stacks were deconvolved using a commercially available inverse filter algorithm (ImagePro). In some cases, stacks of confocal images were obtained.

2.4. Analysis

Images of NMJs were analyzed at high magnification (100x) to determine the extent to which presynaptic SMI+SV2 labeling overlaid postsynaptic labeling for AChRs in superimposed images. SMI+SV2 labeling was used to assign the following categories of endplate innervation; endplates that exhibited a complete absence of SMI+SV2 labeling were considered to be denervated; endplates that showed SMI+SV2 labeling present in all ACHR-rich endplate arms or branches were considered to be innervated; endplates that showed SMI+SV2 labeling present in some but not all ACHR-rich arms or branches were considered to be partially innervated. For analysis, randomly selected fields of NMJs were first located at low magnification. Then, at high magnification all the NMJs in each field were categorized as described above. The extent of endplate S100 coverage was also assessed at 100x magnification and assigned into 3 categories (full, partial, absent). Additional analyses to assess colabeling for P75, S100 and DAPI labeling at 100x magnification were performed as described in Results. Unless noted otherwise, all NMJ measurements were sampled from 80 NMJs per muscle, and only one MG-soleus pair from the same side was sampled per animal.

TSC cell bodies were identified by S100 and DAPI nuclear colabeling (Love and Thompson, 1998). As shown in Fig. 1, multiple DAPI-labeled nuclei are located in the vicinity of the NMJ, so TSC cell body identification needs to be performed with care. To identify TSC cell bodies, multiple focal planes were viewed using a 100x, oil-immersion objective with a variable aperture closed down to minimize depth of focus. At each focal plane, the appropriate fluorescence filters were manually switched while viewing images on a video monitor to ensure that S100 and DAPI labeling were located in identical focal planes. Since some time was needed to accomplish these views, mercury lamp illumination was used to minimize the amount of light exposure. In most cases, these steps were sufficient to either identify TSC cell bodies or to exclude their presence. In more difficult cases, full image stacks of DAPI and S100 labeling were taken to enable finer scrutiny of labeling placement.

2.5. Statistics

Two-way contingency tables were used to determine effects of genotype (B6.SOD1 v WT), age or treatment on frequency distributions of categorical variable means describing innervation status (SMI-SV2 labeling), NMJ S100 and P75 labeling coverage, and S100-P75 colabeling status. For comparing average values, nested analysis of variance was used. Tukey's honestly significant difference post-hoc test was used to test the significance of differences between WT, contralateral control and B6.SOD1 average values. All analysis was performed using commercially available software (Systat Inc). Mean values are presented ± 1 SEM.

3. Results

Previous studies have shown that in $SOD1^{G93A}$ mice, denervation and motor unit dysfunction appear first in muscles that contain fast-type motor units (Frey et al., 2000; Pun et al., 2006; Hegedus et al., 2007). In the gastrocnemius muscles, denervation was found to commence first on fibers belonging to type FF units, while fibers belonging to type S or slow units appeared more resistant to denervation (Frey