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Neuromuscular Junction Morphology and Gene Dysregulation in the Wobbler Model of Spinal Neurodegeneration

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset neuromuscular disease for which there is currently no effective treatment. The progression of ALS includes loss of motor neurons controlling the voluntary muscles, with much of this loss occurring at the neuromuscular junction. In an effort to better understand changes at the neuromuscular junction, we utilized the wobbler mouse model of motor neuron loss. We examined biceps and end plate morphologies and monitored selected factors involved in end plate function. Structural volumes were determined from 3D reconstructions that were generated for the end plates. Wobbler mice exhibited size reductions of both the muscle fibers and the end plates within the biceps, and we found that the end plate volumes were the most sensitive indicator of the degeneration. Concurrently, we found increases in calcitonin gene-related peptide (CGRP) and its receptor in wobbler biceps and spinal cord. We also found increases in gene expression of two acetylcholine receptors within the wobbler biceps, which may be a result of altered CGRP/CALCRL (calcitonin receptor-like receptor) expression.

Keywords

Neuromuscular junction; End plate; Calcitonin gene-related peptide (CGRP); Motor neuron disease; Wobbler mice

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Compliance with Ethical Standards

All experiments were performed in accordance with local and national guidelines.

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Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disease that involves the selective loss of the neurons that control voluntary muscles. This results in a reduction in muscle mass and the ultimate loss of motor control. With no current effective treatment, ALS is fatal, with 50% of patients dying within 30 months and only 15–20% surviving longer than 5 years after diagnosis (Talbot 2009). ALS is the most common adult-onset motor neuron disease, with a prevalence rate of approximately 4.3 per 100,000 population within the USA (Mehta et al. 2016). The causes of ALS and other spinal cord neurodegenerative disorders are largely unknown and there are few current treatment options, which are able to deliver only modest improvements in symptoms (Zarei et al. 2015). Understanding the underlying mechanisms that contribute to the progression of motor neuron disease is essential to the development of new and effective treatments.

Dysregulation and accumulation of proteins is a hallmark of many neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and frontotemporal dementia (Taylor et al. 2002). Aggregation of ubiquitinated cytosolic proteins, such as fused in sarcoma (FUS) (Kwiatkowski et al. 2009) and TAR DNA binding protein (TDP-43) (Neumann et al. 2006), within the motor neurons also occurs in ALS (Blokhuis et al. 2013). Dysregulation of proteins within the neuromuscular junction may also be a contributor to loss of the connected motor neurons. One such protein that is found within the neuromuscular junction is calcitonin gene-related peptide (CGRP). The CGRP pathway is important in many central and peripheral nervous system processes including within motor neurons where CGRP has trophic factor effects. CGRP is also involved in the regulation of acetylcholine receptor (AChR) and acetylcholinesterase (AChE). CGRP levels, important in aging, can be regulated by the cAMP response element binding protein (CREB) (Freeland et al. 2000). In familial ALS patients, CGRP is found to be hyperaccumulated in the anterior horn of the spinal cord (Kato et al. 1991). CGRP is normally expressed in motor neurons at the neuromuscular junction (Mora et al. 1989; Popper and Micevych 1989; Takami et al., 1985a; Takami et al., 1985b) and exerts its effects through binding to the calcitonin receptor-like receptor (CALCRL) in the muscle end plates (Fernandez et al. 2011). CGRP is also responsible for the regulation of AChR synthesis in muscle, which may play a role in muscle contraction (Fernandez et al. 1999; Takami et al., 1985a). Moreover, an increase in expression of AChR has been observed in the muscle of several models of motor neuron degeneration (Fontaine et al. 1987; Fontaine et al. 1986; Melki et al. 1991; Ulbrich et al. 2002), further supporting the potential role of CGRP in the progression of motor neuron loss disorders.

One of the first animal models of familial ALS studied was the superoxide dismutase 1 (SOD1) mouse model. These mice have a smaller body mass, heart, and skeletal muscle mass and AChR mRNA dysregulation in the muscles when compared to wild-type (wt) mice (Kostrominova et al. 2007). In these mice, the loss of the neuromuscular junction occurs followed by axonal degradation resulting in muscle atrophy (Narai et al. 2009). Additionally, CGRP mRNA levels in SOD1 mice were found to be predictive of their vulnerability to degeneration (Ringer et al. 2012). A recent study also showed that disruption of CGRP

accelerates denervation of muscle, while dampening neuroinflammation in SOD1 mice, suggesting a possible dual role of CGRP in the pathogenesis of ALS (Ringer et al. 2017).

Although clinically indistinguishable from familial ALS, most ALS cases are spontaneous with unknown cause. The wobbler mouse represents a model of spontaneously developing ALS-like neurodegeneration. Wobbler model mice (wr) possess a single missense mutation in the vascular vesicular protein sorting gene Vps54 that results in severe motor neuron deficits (Schmitt-John et al. 2005). The wobbler mouse suffers muscular atrophy that results from motor neuron degeneration in the ventral horn of the spinal cord (Baulac et al. 1983; Duchen and Strich 1968; Rathke-Hartlieb et al. 1999; Ulbrich et al. 2002). Our group has previously identified elevated levels, aggregates, and C-terminal fragments of TDP-43 within the wobbler spinal cord (Dennis and Citron 2009) matching that seen in the most common forms of ALS. The potential role that accumulation of CGRP may play in the wobbler phenotype were examined. We evaluated the degree of decreased end plate volumes and increases in CGRP, CALCRL, and AChRs within wobbler muscles and spinal cord. These findings support the idea that protein accumulation within the motor end plates may be driving the development and progression of neurodegeneration in the spinal cord and that the end plate volume may a very sensitive indicator of the course of the disorder.

Experimental Procedures

Animals

Wobbler mice were bred in-house and were originally obtained from frozen embryos from the collection of Dr. Carl Hansen (NIH) and rederived by Jackson Laboratories (Bar Harbor, ME). Mice were maintained under a constant 12 h light/dark cycle, at a consistent temperature of 22 °C ± 0.5 °C. Food and water were supplied ad libitum. All experiments were performed in accordance with local and national guidelines.

Slide Preparation/Staining

At 35 days of age, mice were anesthetized with 3% isoflurane and perfused with 30 ml ice-cold phosphate-buffered saline followed by 30 ml of 4% paraformaldehyde. Spinal cord and muscles were dissected and immersed in 4% paraformaldehyde for 24 h. Tissues were then dehydrated in 15% sucrose for 24 h followed by 25% sucrose for 24 h. Tissues were imbedded in OCT, and 80-µm sections were collected on a Sakura Tissue Tek Cryo3 Cryostat (Torrance, CA) at – 20 °C.

Sections were stained with 500 ng/ml Hoescht 33342, 0.2 U/ml Phalloidin Alexa Fluor 488 (Invitrogen/ Molecular Probes A12379), and 1 µg/ml α-bungarotoxin Alexa Fluor 647 (Invitrogen/ Molecular Probes #B35450) for 60 min. Stain was removed, sections were washed in phosphate-buffered saline, and cover slips were placed using ProLong Gold antifade reagent (Invitrogen/Molecular Probes #P36934).

Microscopy

Images were captured with a Fluoview 1000 laser scanning confocal microscope (Olympus, Pittsburgh, PA). For × 100 magnification (1.40 N.A.), Z-series were collected at 512 × 512

pixel frames at 20 $\mu\text{s}/\text{pixel}$ with sequential laser illumination at 405, 488, and 635 nm to collect Hoechst 33342, Phalloidin-Alexa488, and α -bungarotoxin-Alexa647 fluorescence, respectively. Magnification images ($\times 10$) were similarly collected except with a 0.4-N.A. non-oil objective, and $\times 40$ magnification images were collected at 1024×1024 pixels per frame and 2 $\mu\text{s}/\text{pixel}$ with a 1.3-N.A. oil objective.

ImageJ was used for all analyses of the micrographs (Schneider et al. 2012). Muscle fiber diameters were determined by measuring at least ten fiber diameters per bicep by drawing measured perpendicular lines across the fibers at their maximum diameter in $10\times$ z-stacks.

End Plate Analysis

End plate volumes were determined with the aid of the 3D Object Counter plugin (Bolte and Cordelieres 2006) examining the $40\times$ Z-stacks. After installation of the 3D Objects Counter, 3D ROI Manager, and 3D Viewer plugins, the original Z-series.oib file was opened as a hyperstack in default and autoscale modes without splitting channels. The Image-Color-Split Channels command was used to isolate the channel containing the end plate data. The end plate only stack was then converted to 8-bit maintaining proportionality of voxel intensities. After choosing the appropriate parameters in Set 3D Measurements, the 3D Object Counter was run with a threshold intensity of 50 and 100 minimum voxels for any object. Objects on edges were excluded and connected voxels were grouped, numbered, and color coded. Approximately 25 end plates in each stack were generated and analyzed for the size parameters. 3D renderings of the end plates were obtained after conversion of the color coded object stacks to RGB with the Image-Stacks-3D Projection command. Examples of the end plate fields in the z-series collected from the Hoechst 33342, phalloidin-Alexa488, and α -bungarotoxin-Alexa647 are shown in the supplementary movies for the wild-type (S 1A) and wobbler (S 1B) generated displaying brightest points and rotating in 10° increments completely around the y-axis with interpolation. Supplementary movies S 2a and S 2b display the isolated end plate structures for the wild-type and wobbler, respectively.

Western Immunoblotting

Total protein was extracted from tissue using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% triton, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM tris-HCl pH 7.4). Protein was quantified using Pierce BCA protein assay (Pierce/Thermo Scientific, Waltham, MA) and read on a BioTek (Winooski, VT) microplate reader at 570 nm. Protein was denatured by heating to 95°C for 5 min and separated on a gradient SDS page gel. The gel was transferred onto a nitrocellulose membrane, blocked using Odyssey blocking buffer (LI-COR, Lincoln, NE), immunoblotted overnight with antibodies, and visualized using a LI-COR odyssey fluorescence scanner. The following antibodies were used: CRLR (CALCRL) (sc-30028) rabbit anti-mouse (Santa Cruz Biotechnologies, Santa Cruz, CA), GAPDH (MAB374) 1:1000 anti mouse (Chemicon Int., Temecula, CA), and HSP70 (sc-33575) 1:1000 anti-rabbit (Santa Cruz Biotechnologies, Santa Cruz, CA).

qRT-PCR

RNA was isolated from tissue using RNeasy columns (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA was quantitated using a Nanodrop 2000 (Thermo

Scientific, Rockford, IL) and was amplified using Quantitect SYBR RT-PCR kit (Qiagen, Valencia, CA). Samples were amplified according to manufacturer's recommendations. The reaction was initiated at 95 °C for 10 min, then denatured at 95 °C for 15 s, annealed at °C for 30 s, and extended at 72 °C for 30 s for a total of 40 cycles. Primer pairs, synthesized by IDT (Coralville, IA), were taken from published examples, e.g., Human GAPDH (Zhang et al. 2007), and are shown in Table 1.

Statistics

Mean values are depicted $\pm$ standard error and wobbler samples were compared with wild-type by the Mann-Whitney test with $p < 0.05$ indicating significance.

Results

Wobbler Mice Present Decreased Fiber Diameters and End Plate Volumes

To further understand the selective motor neuron loss that wobbler experience and resulting muscle atrophy, we examined the muscle fibers and motor neuron end plates in the biceps of the wobbler mouse. Average fiber diameter was decreased in the wobbler mouse by approximately 30% when compared to their wild-type littermates (Figs. 1a, b and 2a). In addition, motor neuron end plates in wobbler mice were reduced 4-fold in volume (Figs. 1c, d and 2b). Full 360° rotations of the end plates and the end plate objects are shown in the videos in Supplementary Fig. S1a–d and enable evaluation of the full confocal information collected to determine the end plate morphology.

CGRP Expression Increased in Wobbler SC

To characterize the mechanism of motor neuron death and decreased end plate volume, we evaluated the expression of CGRP within the spinal cord neurons. We observed an increase in CGRP mRNA expression in the spinal cord of wobbler mice compared to control littermates as measured by qRT-PCR (Fig. 3a). CGRP expression was not detectable in the biceps of wobbler or control mice.

CALCRL Protein Increases in Biceps Trend in SC

Western blots were used to analyze the protein expression of CALCRL in both the spinal cord and biceps of wr and wt mice at 35 days of age (Fig. 3b). The expression of the receptor for CGRP was found to be increased by approximately 2.6-fold within the wobbler mice when compared to wild-type litter-mates. In addition, an increasing trend of approximately 2-fold was found within the spinal cord.

Expression of Acetylcholine Receptors Increase in Wobbler Biceps

In order to assess any effects that an increase in CGRP expression had on acetylcholine receptors, we utilized qRT-PCR to evaluate the expression of AChRs within the biceps of wildtype and wobbler mice. We found that AChR α_1 mRNA expression was upregulated 18-fold and AChR δ mRNA expression was upregulated 15-fold (Fig. 4) in the biceps of wobbler mice. AChR β_1 and AChR ϵ showed no significant difference between groups. In addition, there was no significant difference in mRNA expression of acetylcholinesterase

(AChE) in wobblers biceps compared to wild-type. In sum, we do see an increase in some AChRs which may be the result of an increase in CGRP expression in the wobbler mouse.

Discussion

ALS is the most common adult-onset motor neuron disease; there are currently very few treatments offering modest improvement, and the cause is largely unknown (Mehta et al. 2016). Protein dysregulation is a hallmark of many neurodegenerative disorders and has been observed in ALS (Blokhuys et al. 2013). In particular, we hypothesized that dysregulation of proteins within the neuromuscular junction may contribute to the development and progression of ALS symptoms. We utilized the wobbler mouse model of motor neuron disease to better understand the structural alterations and the potential effect of the neuromuscular protein, CGRP, and related factors.

In order to confirm the presence of an ALS-like phenotype in our mouse model, we evaluated muscle atrophy within the bicep. Our wobbler mouse model displayed significant muscle atrophy, as measured by a decrease in bicep diameter when compared to wild-type mice. This finding is consistent with atrophy observed previous wobbler studies (Duchen and Strich 1968) and with ALS patients (Talbot 2009). We found that mouse muscle fiber diameters were reduced 32% in our wobbler mice at 35 days of age compared to the wild-type littermate muscle fiber diameters of 17.3 μm . This is consistent with a 57% reduction to 15 μm observed at 70 days after birth in a C57Bl/6 background (Hantai et al. 1995) and a 29% reduction seen in 42-day-old wobbler mice, compared to wildtype controls (Oda 1985; Vergani et al. 1997).

We found that the receptor for CGRP, CALCRL, was increased in both biceps and spinal cord of wobbler mice when compared to wild-type mice. In addition, we saw an increase in CGRP expression within the spinal cord. This is consistent with reports of CGRP in other ALS model mice (Vlug et al. 2005) and could involve aberrant trafficking and/or transcription factor elevation, e.g., ATF3 or c-Jun (Kato et al. 1991). Future colocalization studies could address these interactions. The increases in both receptor and ligand in ALS model mice suggest that CGRP may be playing a role in the pathogenesis of ALS symptoms through its accumulation at the neuromuscular junction.

CGRP has been shown to be responsible for the regulation of AChR synthesis in muscle (Takami et al., 1985b), and increases in expression of acetylcholine receptors (AChRs) have been observed in the muscle of several models of motor neuron degeneration (Fontaine et al. 1987; Fontaine et al. 1986; Melki et al. 1991; Ulbrich et al. 2002). In our study, we observed an increase in mRNA expression of two AChRs, AChR α_1 and AChR δ , in wobbler biceps.

In the wobbler mouse model of ALS, we have identified CGRP as a potential factor in the progression of motor neuron degeneration, acting at the neuromuscular junction. We have shown an increase in CGRP mRNA expression in the wobbler spinal cord, concurrent with an increase in CALCRL protein expression in the bicep. Increased expression of both CGRP and its receptor may also lead to increases in the expression of AChRs within the muscle. This demonstrates upregulation of CGRP in the wobbler mouse model of motor neuron loss

and is consistent with the previous studies showing a similar finding in the SOD1 model of familial ALS (Ringer et al. 2017; Ringer et al. 2012). As with the SOD1 study, CGRP may be playing a dual role in the pathogenesis in the wobbler mouse. Further study into the role of CGRP in this model will help to identify the mechanism by which it may be contributing to sporadic neurodegeneration and how we may be able to target this mechanism for treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Bicep muscle fibers were stained for nuclei (Hoechst 33342, blue channel), F-actin (phalloidin Alexa488, green channel), and acetylcholine receptors (α -bungarotoxin Alexa647, red channel). It can be seen in the maximum z projections of the confocal slices that, compared with the wildtype (a) and the wobbler (b) fiber diameters, indicate atrophy. The end plate structures in the wild-type (c) muscle display normal neuromuscular junctions while the wobbler end plates (d) are significantly reduced and less organized. End plate volumes were determined by isolating the bungarotoxin positive objects in 3D. An example

volume is noted by the arrow in **c**, and examples of the corresponding computer generated end plates are shown below **c** and **d**. Supplementary videos S1A, S1B, S2a, and S2b provide 3D rotations of the end plates and the equivalent objects

Fig. 2.

Muscles from wild-type (wt) and wobbler (wr) mice were analyzed at 35 days of age. **a** Diameters were measured for 10 fibers per muscle from each genotype ($n = 3/\text{group}$) and were found 30% reduced in the wobbler. **b** The volumes were determined for every end plate in each biceps muscle sample ($n = 4/\text{group}$) and were found decreased by 75% in the wobbler mice (* $p < 0.05$)

Fig. 3.

CGRP and CALCRL expression in spinal cord and biceps. **a** CGRP mRNA was significantly increased ($*p < 0.05$) in wr spinal cords at 35 days of age ($n = 9$) compared to wt controls ($n = 8$). CGRP mRNA expression was not detected in biceps. CALCRL mRNA expression analysis revealed a trend toward higher expression in wr spinal cord and lower expression in wr biceps, though this was not statically significant. **b** In contrast, CALCRL protein was found elevated in the biceps. Western Blots were performed using an antibody for CALCRL on tissue harvested from wr and wt mice at 35 days of age. Expression of CALCRL was found to be increased in wr mice in both **a** spinal cord ($n = 9$) and **b** biceps ($n = 5$) ($***p < 0.0001$)

Fig. 4.

The mRNA expression of several acetylcholine receptor subtypes was evaluated by qRT-PCR of RNA isolated from the biceps. There were no detectable changes for AChR β_1 , AChR ϵ , and AChE (not shown). Significant differences were only found for AChR α_1 and AChR δ , and these mRNAs were both elevated in the wr biceps ($p < 0.05$)

Table 1

Primer sequences used for qRT-PCR are all shown in 5' → 3' orientation

Gene	Accession	Upstream/downstream	Position/length
AChR α_1	NM007389.4	TAACGCCGCTGAGGAATGGAAGTA / TCAGCCCTCTGCTCATCCTTGTGA	1258–1404, 147 bp
AChR β_1	NM009601.4	ATAGCTCGGTGAGGCCGGCG / TAGGTGACCTGGATGCTGCA	174–528, 355 bp
AChR δ	NM021600	AGGACAAAGTGGATGTCGCCCTAA / AGCCCCGAGTCATAGACAAAGCACAT	194–460, 267 bp
AChR ϵ	NM009603.1	ACAATGCTGAGGAGGTGGAGTTCA / TGGCCCCATTCTCCATTCTCGGTAA	531–638, 108 bp
AChE	NM009599	AGCTGATAGCCTTGCTTGAGGACAA / ATTGTCTTTTGTCTGAAGCCTGGGAC	1010–1257, 248 bp
CALCRL	NM018782	GGCCAAATAACCAAGGCCTTAG / CAGCCCATCAGGTAAAGATG	1108–1188, 81 bp
18S rRNA	X00686	GCTCGCTCCTCTCCTACTTG / CCCTCTCCGGAATCGAAC	126–439, 314 bp