Motor Unit Characterization in Canine Degenerative Myelopathy: A Disease Model for
Amyotrophic Lateral Sclerosis

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The undersigned, appointed by the dean of the Graduate School,
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MOTOR UNIT CHARACTERIZATION IN CANINE DEGENERATIVE MYELOPATHY: A DISEASE
MODEL FOR AMYOTROPHIC LATERAL SCLEROSIS

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A candidate for the degree of

Biological Sciences

And hereby certify that, in their opinion, it is worthy of acceptance.

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Table of Contents

Acknowledgements.....	ii
List of Tables	vi
List of Figures	vii
ABSTRACT.....	1
CHAPTER 1 General Overview	3
The Motor Pathway	3
Amyotrophic Lateral Sclerosis.....	7
Superoxide Dismutase 1.....	10
<i>Normal SOD1 Structure and Function</i>	10
<i>Mutant SOD1 Toxicity in ALS</i>	12
SOD1 Transgenic Mouse Models	16
FALS associated with the D90A missense mutation in <i>SOD1</i>	18
Canine Degenerative Myelopathy (DM)	20
CHAPTER 2 Characterization of Intercostal Muscle Pathology.....	23
CHAPTER 3 Characterization of Thoracic Motor and Sensory Neurons and Spinal Nerve Roots	50
CHAPTER 4 General Discussion	74
CHAPTER 5 Future Directions	81
REFERENCES	89
VITA.....	102

List of Tables

Table 1. Requirements for ALS diagnosis.....	8
Table 2. Clinical grading scale of disease progression in DM-affected dogs	21
Table 3. <i>SOD1</i> genotypes	37
Table 4. Summary of the number of dogs within each disease grade	56
Table 5. <i>SOD1</i> genotypes	56
Table 6. PWC T8 motor root morphometric measurement results	63
Table 7. Boxer T8 motor root morphometric measurement results.....	64
Table 8. Histopathological assessment motor root myelinated axons	65
Table 9. PWC morphometric sensory root data	69
Table 10. Histopathological assessment sensory root myelinated axons.....	71
Table 11. Comparative summary of human ALS and ALS disease models	80

List of Figures

- Figure 1. Anatomy of a motor unit. A motor unit consists of (1) a motor neuron, (2) its axon, and (3,4) the myofibers it innervates. Courtesy of Dr. Joan Coates. 4
- Figure 2. SOD1 protein sequence homology between humans, mice, and dogs (Green et al. 2002) 11
- Figure 3. Normal SOD1 structure and function (Boissinot et al. 1997). (A) SOD1 has a Greek key topology, named so because diagrams depicting the connections of the 8 antiparallel strands resemble designs found on ancient Greek pottery. (B) Strands are twisted into a “ β -sandwich” with 4 strands comprising each half of the sandwich. The 2 halves are covalently bound by 2 Greek key loops found at the ends of the barrel, and each monomer contains one copper and one zinc involved in maintaining structure and function. (C) Normal enzymatic function involves the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen..... 13
- Figure 4. DM affected spinal cords display SOD1 positive aggregates in motor neurons, and marked myelinated axon loss in white matter. Immunohistochemical demonstration of SOD1-containing aggregates in motor neurons in the thoracic spinal cord (A,B). Numerous aggregates stained with an antibody directed against SOD1 were present in ventral horn motor neurons from a DM-affected PWC (B). Similar aggregates were present in all affected dogs of both PCW and Boxer breeds. These inclusions were not observed in thoracic cord motor neurons from unaffected dogs (representative preparation from a control PWC shown in B). Luxol fast blue and periodic acid-Schiff stained thoracic spinal cord from unaffected (C) and grade 4 (D) PWCs. Pallor of the peripheral white matter in (D) corresponds to axonal loss and demyelination (arrows). Higher magnification of thoracic white matter from unaffected (E) and grade 4 (F) PWCs stained with toluidine-blue. Substantial axonal loss with myelin pathology is seen in the PWC with advanced DM. Bars in (A), (C) and (E) indicate magnifications in (B), (D) and (F) respectively..... 29
- Figure 5. Methods for morphometric analysis of myofibers (A) Hematoxylin and eosin stained intercostal muscle cross sections were imaged with light microscopy at a 200x magnification. (B) Fiji™ or Photoshop™ imaging software were used to generate a black and white segmented mask outlining the boundaries of each myofiber. (C) In Photoshop™, the mask was overlaid on the gray-scaled original image, transparency increased, and manual corrections were made to define the boundaries of each fiber. Once boundaries are defined, the corrected mask is thresholded in Fiji™, and a particle analysis tool was used to obtain

measurements. Measurements include minor axis length, fiber cross sectional area, and roundness. (D) The minor axis is identified as the shortest axis of a best fit ellipse (Arrow). Pixels of this axis and of the cross sectional areas were converted to μm and μm^2 respectively for each myofiber. The percent of angulated fibers (example labeled with “a” in insert of panel C) were determined manually by a masked evaluator. 36

Figure 6. Severe pathology in intercostal muscle of late stage DM dogs Intercostal muscles were stained with H&E (A and C), and Masson trichrome (B and D), to visualize general morphology of myofibers and presence of fibrotic tissue. (A and B) Myofibers from a control PWC display uniform size and shape with no fibrosis seen between fibers. (C) Myofibers from a grade 4 PWC showing variability in size and shape with hypertrophy (long arrow) and atrophy (short arrow) fibers. (D) Late-stage PWC displaying fibrosis (blue stain). 38

Figure 7. Morphometric data reveal increased variability of intercostal myofiber sizes in DM-affected PWC. (A, D) Size distribution graphs of myofibers from affected PWCs show changes in the distribution of fibers sizes (minor axis, and cross sectional area respectively). (B, E) Distribution graph of affected PWCs separated into early (n = 3) and late (n=9) stages. Early stage myofiber distributions were shifted towards larger size classes, while more small fibers were seen in the late stage distribution. (C, F) No significant difference was seen between the mean minor axis lengths or cross sectional area of unaffected and affected dogs; however the range of fiber sizes were significantly increased in affected PWCs (* p<0.05). 40

Figure 8. Boxer morphometric data reveal no significant differences in intercostal myofiber sizes. (A, D) Size distributions of minor axis length and cross-sectional area of myofibers from unaffected and affected samples. (B, E) When affected dog data is separated into early and late stage (early: n=2, Late: n=2), early disease samples exhibit a peak in the medium size range. (C, F) No significant differences were detected in mean minor axis length or range between control and affected groups. 41

Figure 9. Acetylcholine receptor (AChR) complexes remain innervated in DM-affected intercostal muscle. Thoracic intercostal muscle from one unaffected and six affected dogs were stained with alpha bungarotoxin to label AChR’s (red), and anti-NF-L to label axon terminals (green). At least 100 complexes were imaged from each sample. Axons were scored based on proximity to AChR complexes as intact (A), partially intact (B), or absent (C). (D) Bar graph showing the percentage of receptors from each sample that were intact, partially intact, or absent. Insert in A is a higher magnification showing contact between the nerve terminal and the receptor complex. A-C are representative images of observations from control and affected samples. 42

Figure 10. Changes in fiber type patterns in intercostal muscle of late stage DM dogs. Intercostal muscles were stained with anti-myosin heavy chain-1 to visualize the proportions of fiber types. (A) Unaffected PWC with normal checkerboard pattern of type 1 and 2 fibers. (B) Grade 4 PWC with a high predominance of type 1 fibers. Bar in (A) represents magnification in both images. (C) Bar graph displaying % of type 1 myofibers in PWC (left) and Boxer (right) thoracic intercostal muscle. PWC graph correlates with observations, with a larger percentage of type 1 fibers in grade 4 dogs (control n = 1, affected n = 4). (Boxers: control n= 1, affected n =2). 43

Figure 11. Procedure for obtaining morphometric measurements in T8 motor and sensory root cross sections. (A) Resin embedded nerves were sectioned and stained with P-phenylene diamine (PPD), imaged using light microscopy, and composite images of the entire nerve cross-sections were constructed. (B) Magnified image of area outlined in (A). (C) Images were sharpened using Photoshop™ tools. (D) Imaris® software was used to invert the image, and remove background noise. (E) Multicolored image produced by the automated count tool in MetaMorph® software. Colors indicate what software distinguishes as individual objects. Note that several adjacent axons are being mistaken for a single object. (F) Manual corrections were made in Photoshop by overlaying, and increasing the transparency of, the multicolored image over the sharpened light micrograph. (G) Multicolored images after manual corrections. (H) Corrected multicolored image of entire nerve root cross-section. (Illustration represents a T8 motor root cross section)..... 60

Figure 12. Thoracic motor neuron morphometric measurements. (A, B) T7 spinal cord section from an unaffected 9 year old Boxer stained with ChAT. (B). Higher magnification of highlighted box within Rexed lamina 9 in A. This image is a representation of the normal motor neuron morphology seen in unaffected and affected dogs of both breeds. (C) Morphometric measurements obtained from PWC (top) and Boxers (Bottom). Pooled data indicate no difference in the motor neuron density between unaffected and affected samples of either breed (*Affected boxers: one grade 1, three grade 2, one grade 3, and two grade 4 dogs; Affected PWC: two grade 2, two grade 3, and four grade 4 dogs*). Likewise no differences were seen in the mean soma cross sectional area (CSA) or size range. In PWC's a significant difference was seen in the CSA standard deviation, suggesting more variability in soma area in affected dogs ($p=0.02$). One Way ANOVA indicated the difference in standard deviation is attributed to a significant difference between unaffected and late stage (grade 3-4) PWC samples ($p= 0.04$). 62

Figure 13. Motor root myelinated axon diameter distribution from PWC's and Boxers. Motor root myelinated axon diameter distribution from PWC's (A,B) and Boxers

(C,D). Histograms show a bimodal size distribution with one peak representing small caliber axons, and one peak representing large caliber axons (A, C). For both breeds, no obvious differences could be seen between large caliber axons from control and affected motor roots, while the small caliber peak was more variable. (B, D) For both breeds, no statistical difference was seen in the percentage or average numbers of axons $\leq 5 \mu\text{m}$. Data represented in this figure were obtained from the same samples depicted in tables 6 and 7..... 67

Figure 14. PWC sensory root and dorsal root ganglia histopathology. Histological sections of T8 sensory roots (A and B) and dorsal root ganglia (DRG) (C and D) in PWC samples. EM-fixed sensory roots and DRG from control (A,C) and grade 4 (B,D) PWC samples were embedded in resin and stained with PPD and toluidine blue respectively. Compared to controls (A), sensory roots from grade 4 PWC (B) exhibited decreased myelinated axon density. DRG neurons from grade 4 PWC (D) contained numerous cells with condensed cytoplasm (arrowhead) and severely pycnotic nuclei (arrow) that were not present in the DRG neruons from age-matched control dogs (arrowhead and arrow in C denote cytoplasm and nucleus of normal neuron for comparison)..... 68

Figure 15. PWC sensory root myelinated axon diameter distribution. (A)Histogram shows a bimodal size distribution with one peak representing small caliber axons, and one peak representing large caliber axons. (B) There was a statistically significant difference in the average number of axons $\leq 5 \mu\text{m}$ ($p=0.03$) and $>5 \mu\text{m}$ ($p=0.03$). Data represented in this figure were obtained from the same samples depicted in table 9 70

Figure 16. Tidal breathing flow-volume loop. Loops are plotted as volume (mL) vs flow (L/sec). Normal loops resemble a “D” shape. Inspiration begins on the right side and continues clockwise as inspiration ends, and expiration begins (arrows). Changes in loop shape may be indicative of respiratory abnormalities. 82

ABSTRACT

Dogs homozygous for missense mutations in superoxide dismutase 1 (*SOD1*) develop a late-onset neuromuscular disorder called degenerative myelopathy (DM) that has many similarities to amyotrophic lateral sclerosis (ALS). Both disorders are characterized by widespread progressive declines in motor and sensory functions accompanied by atrophic changes in the ascending and descending spinal cord tracts. Additionally, some forms of ALS are also associated with *SOD1* mutations. In end-stage ALS, death usually occurs as a result of respiratory failure due to severe functional impairment of respiratory muscles. The mechanisms that lead to this loss of function are not known. Dogs with DM are euthanized at all stages of disease progression providing an opportunity to characterize the onset and progression of any pathological changes in the respiratory muscles that may precede respiratory failure. To characterize such potential disease-related pathology we evaluated intercostal muscles from Boxer and Pembroke Welsh Corgi (PWC) dogs that were euthanized at various stages of DM disease progression. DM was found to result in intercostal muscle atrophy, fibrosis, and an alteration in muscle fiber type composition. This pathology was not accompanied by retraction of the motor axon terminals from the muscle acetylcholine receptor complexes, suggesting that the muscle atrophy did not result from physical denervation. To determine if other components of the thoracic motor unit (cell body, axon, and myofibers) also demonstrated morphological changes consistent with dysfunction,

histopathologic and morphometric analyses were conducted on thoracic spinal motor neurons (MNs) and in motor root axons from DM-dogs. Because sensory dysfunction is known to occur in DM, dorsal root ganglia (DRG), and sensory root axons were also analyzed. No alterations in MNs, or motor root axons were observed in either breed. However, advanced stage PWCs exhibited significant losses of sensory root axons, and numerous DRG sensory neurons displayed evidence of degeneration. These results indicate that intercostal muscle atrophy in DM is not preceded by physical loss of the MNs innervating these muscles, or of their axons. Axonal loss in thoracic sensory roots and sensory nerve death suggest sensory involvement may play an important role in DM disease progression. Results from this investigation support previous findings that DM is a multisystem disease with many similarities to human familial *SOD1*-D90A ALS, indicating that DM is a suitable disease model for this form of ALS. Further analysis of the mechanisms responsible for these morphological findings would aid in the development of therapeutic intervention for both diseases.

CHAPTER 1

General Overview

The Motor Pathway

Amyotrophic lateral sclerosis (ALS) and canine degenerative myelopathy (DM) are diseases that affect the central and peripheral nervous systems. The central nervous system is composed of the brain and spinal cord, and the peripheral nervous system includes cranial and spinal nerves. Motor neurons (MNs) such as those found in the motor cortex and brainstem (upper motor neurons [UMNs]) possess axons that descend through the spinal cord white matter (i.e. corticospinal, rubrospinal, vestibulospinal, reticulospinal tracts), and synapse onto interneurons and MNs within the brain stem and ventral horn of the spinal cord segments. MN axons of the spinal cord exit the spinal cord as ventral (motor) roots and merge with dorsal (sensory) roots at the intervertebral foramen to form the spinal nerves.

A motor unit (MU) consists of a MN cell body, axon, and the associated myofibers (Figure 1). The MU functionally is termed lower motor neuron (LMN). Alpha MNs have large somas, and innervate extrafusal myofibers. Within the alpha MN population exists a range of soma sizes and corresponding axon calibers. Generally larger MN's have larger caliber axons, and innervate fast twitch myofibers. They are involved in producing quick bursts of powerful muscle contractions, and are quick to fatigue. Conversely, smaller MNs have smaller caliber axons that innervate slow twitch myofibers and are fatigue resistant. MU's

Figure 1. Anatomy of a motor unit. A motor unit consists of (1) a motor neuron, (2) its axon, and (3,4) the myofibers it innervates. Handbook of Veterinary Neurology, 5th ed. Lorenz MD, Coates JR, Kent M.

can be further defined by size (i.e. compared to larger MNs, smaller MNs innervate fewer, thinner, myofibers and therefore comprise smaller MUs) and metabolic properties. Since slow-twitch fibers have large numbers of mitochondria, myoglobin, and oxidative enzymes, the aerobic metabolism makes them fatigue resistant. These myofibers express the type 1 myosin isoform (type 1 myofibers) (Swynghedauw 1986). Fast twitch myofibers express the type 2 myosin isoform (type 2 myofibers) (Swynghedauw 1986), and utilize glycogen, whereby anaerobic catabolism makes them susceptible to fatigue (specifically type 2B). The intermediate type 2A myofibers are fast twitch /fatigue resistant with the capacity to undergo aerobic catabolism. Labeling cross sections of muscles can identify the distribution of myofiber types, which even within the same individual varies between different muscles, and within the same muscle

groups (Johnson et al. 1973). Cross sections of muscles with equivalent amounts of each type normally display a “checkerboard” pattern (Armstrong et al. 1982). In denervated muscle, the distribution of myofiber types may undergo fiber typed grouping. These denervated myofibers become reinnervated by neighboring axons, and accordingly express the myosin isoform dictated by its new MN innervation (Buller et al. 1960). The histochemical staining result is groups of myofibers of the same type. In situations where the MN is unable to reinnervate there may be a lack of fiber typed grouping. Thus the affected muscle would display groups of myofibers of mixed types. Another histological abnormality is the presence of groups or clusters of angular atrophic myofibers that develop as a result of denervation, and the scalloped profile is a result of pressure from surrounding hypertrophic and/or normal myofibers. Muscle pathology is also heavily involved in disease primarily affecting myofibers (i.e. muscular dystrophies) (Sewry 2010). Classic features of myopathic disease like muscular dystrophies include diffuse variability of myofiber sizes of both fiber types, necrosis, extensive fibrosis, and fiber regeneration (Sewry 2010). It has been demonstrated that in some hereditary forms of ALS mutant gene expression within myofibers has a dominant role in disease pathology (Dobrowolny et al. 2008; Wong and Martin 2010). Therefore, it is important to distinguish between neuropathic and myopathic histopathology.

In ALS and DM, voluntary motor control is disrupted (Averill 1973; Charcot J-M 1869). Voluntary movement is achieved by the convergence of information from motor and sensory pathways, via spinal reflexes, and disruption of

components and modulators of the reflex arc cause characteristic phenotypes important in diagnosis of neurological disorders. Spinal reflexes are unconscious and involuntary feedback circuits in which sensory information is relayed to spinal MNs, which in turn stimulate the muscle to respond accordingly. For example when a muscle is stretched, primary large caliber afferent axons in muscle spindles, Ia sensory fibers, become depolarized (Liddell and Sherrington 1924). Afferent sensory fibers, which synapse onto MNs and other Ia inhibitory interneurons. As a result, MNs of the stretched muscle are excited, and those of the antagonist muscle are inhibited through reciprocal inhibition. Consequently, the stretched muscle contracts, and the opposing muscle relaxes in an attempt to maintain normal length (Liddell and Sherrington 1924). Pathology of any of the above reflex components leads to decreased and/or loss of spinal reflexes. Additionally, clinical evidence of MU disruption (LMN signs) includes muscle weakness, decreased muscle tone, flaccid paralysis, and neurogenic muscle atrophy. Spinal reflex evaluations (reduced or absent) are used to localize LMN lesions.

Spinal reflexes are modulated by the UMN pathways. These pathways originate in the cerebral cortex and brainstem nuclei and control specific tasks. UMNs and relay pathways synapse directly onto MNs or interneurons (i.e. inhibitory interneurons) in the spinal grey matter. Thus, descending UMN pathways can mediate excitation of specific MN pools controlling contraction and relaxation of muscle groups according to the intended movement. Disruptions of these pathways lead to UMN signs including spasticity (increased muscle tone

and resistance to stretch), slowing of movements (spastic paresis), disuse atrophy and hyperreflexia. Presence of UMN and LMN signs and spread of signs to other regions within the CNS are used in the clinical diagnosis of ALS and DM, and for evaluation of disease progression (Brooks et al. 2000; Coates and Wininger 2010).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common group of adult motor neuron diseases (Cleveland and Rothstein 2001). There are many variants of ALS, but they all have a number of features in common. ALS is generally characterized by progressive muscle wasting, resulting in paralysis and death due to respiratory muscle failure (Charcot J-M 1869). Typically ALS affects adults 50 years and older, and patients generally succumb to the disease process ~3-5 years after onset. There is no cure for any form of ALS, and currently the only FDA approved drug is Riluzole, a drug that provides minimal functional benefit to patients (Bensimon et al. 1994). Riluzole is believed to primarily assist in modulating glutamate-mediated excitotoxicity (Mizoule et al. 1985). Population studies in the U.S.A. indicate ~5,600 people are diagnosed per year (15 cases/day), and at any given time ~30,000 Americans are living with this disease (Hitsumoto et al. 2006). ALS is 20% more prevalent in men, and 93% of patients in the ALS CARE database are Caucasian (Hitsumoto et al. 2006).

Based on the El Escorial-Revised diagnostic criteria, a definitive diagnosis of ALS includes clinical evidence of LMN and UMN dysfunction, and progressive

Table 1. Requirements for ALS diagnosis

The presence of	Evidence of LMN degeneration by clinical, electrophysiological or neuropathologic examination
	Evidence of UMN degeneration by clinical examination
	Progressive spread of symptoms or signs within a region or to other regions
The absence of	Electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration
	Neuroimaging evidence of other disease processes that might explain observed clinical and electrophysiological signs.

spread of symptoms or signs within a body region and to other body regions that cannot be explained by other disease processes (Table 1) (Brooks et al. 2000). ALS is further classified based on predominance of signs at clinical onset (limb verses bulbar), the involvement of LMN and UMN signs at onset (LMN onset, UMN onset, bulbar onset), and the rate of progression (rapid, slow)(Kiernan et al. 2011; Ravits and La Spada 2009). In some ALS patients, abnormalities of sensorium has been reported, with symptoms including burning, pain, paresthesia, numbness and decreased vibration sensation (Andersen et al. 1996; Isaacs et al. 2007; Kawamura et al. 1981; Mulder et al. 1983). Because individuals exhibit various combinations of the above stated clinical features, clinical phenotype is heterogeneous (Brooks 1994; Brooks et al. 2000). ALS is also genetically heterogeneous (Al-Chalabi et al. 2012). 5-10% of ALS cases are familial (inherited) and the remaining are sporadic with no family history (Mulder et al. 1986). Of the familial forms many genes have been identified as causative

including SOD1 (Rosen et al. 1993), TARDBP (Gitcho et al. 2008), FUS (Laaksovirta et al. 2010), C9orf72 (DeJesus-Hernandez et al. 2011), and UBQLN2 (Deng et al. 2011). Because of the clinical and genetic heterogeneity associated with ALS, it is believed to be a group of multisystem diseases that involve both motor and sensory systems (Hays et al. 1991).

Histological evaluations of human ALS tissues have identified distinguishing neuropathologic features (hallmarks) indicative of MU degeneration. As the term “amyotrophic” implies, muscle wasting is a prominent characteristic feature observed in all ALS patients (Charcot J-M 1869), and muscle biopsy studies reveal neurogenic muscle atrophy (Andersen et al. 1996; Baloh et al. 2007; Fischer et al. 2004; Soraru et al. 2008a; Telerman-Toppet and Coers 1978). It has been postulated that the pattern of myofiber type composition varies in different forms of ALS (Soraru et al. 2008a). In reports from individuals with UMN dominant ALS and individuals with a protracted disease course exhibit fiber typed grouping (Andersen et al. 1996; Soraru et al. 2008a), while other forms of the disease exhibit general atrophy of groups of fibers (Baloh et al. 2007).

A major histopathological feature seen in MUs of ALS patients at end stage disease is spinal MN soma loss (Ravits et al. 2007; Wetts and Vaughn 1996). Surviving MN somas have been noted to display abnormal inclusion bodies/aggregates (i.e. Bunina bodies, Lewy body-like inclusions, and hyaline inclusions) (Hirano 1991), and morphological features of programmed cell death (Martin 1999). Additionally, ALS patients with mutations in superoxide dismutase

1 (*SOD1*) have aggregates which label positive with antibodies against SOD1 (Bruijn et al. 1998; Forsberg et al. 2010; Shibata et al. 1996). Drop out of myelinated axons, particularly large caliber axons, in motor roots (Hanyu et al. 1982; Kawamura et al. 1981; Sobue et al. 1981) and spinal nerves (Giannini et al. 2010; Perrie et al. 1993) has also been reported.

Superoxide Dismutase 1

Mutations in superoxide dismutase 1 (*SOD1*) account for 20% of familial ALS cases (Rosen et al. 1993). To date, over 160 mutations in *SOD1* have been recorded as a cause of ALS, spanning all five exons of the gene (<http://alsod.iop.kcl.ac.uk/>). Large efforts have been put into generating transgenic rodent models that over express *SOD1* mutations for study to provide insights into the phenotype and pathogenic mechanisms of ALS.

Normal SOD1 Structure and Function

Superoxide dismutase 1 (SOD1) is a member of an enzyme family that works to counter oxidative stress caused by normal cellular metabolism (Fridovich 1975; Johnson and Giulivi 2005). This ubiquitously expressed cytoplasmic protein has a molecular weight of ~32,000 daltons, consists of 153 amino acids, and is known as one of the most stable proteins (Fridovich 1975; Fridovich 1986; Green et al. 2002; Johnson and Giulivi 2005; McCord and Fridovich 1969; Rakhit and Chakrabartty 2006). The SOD1 protein sequence is highly conserved across numerous species, including 79% amino acid identity between the human, mouse and canine proteins (Figure 2) (Bannister et al.

human sod1	MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTS	60
mouse sod1	MAMKAVCVLKGDGPVQGTIHFEQKASGEPVVLGGQITGLTEGQHGFHVHGYDNTQGCTS	60
canine sod1	MEMKAVCVLKGGPVEGTIHVFQKSGS-PVVVSGTITGLTEGEHGFHVHQFGDNTQGCTS	59
	* * * * *	
human sod1	AGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVAADVSIEDSVISLSGDHCCIIGRTLTVV	120
mouse sod1	AGPHFNPHSKKHGGPPADEERHVGDLGNVTAGKDGVANVSIEDRVISLSGEHSIIIGRTMVV	120
canine sod1	AGPHFNPLSKKHGGPKDQERHVGDLGNVTAGKDGVAIVSIEDSLIALSGDYSIIIGRTMVV	119
	* * * * *	
human sod1	HEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQXTFP	158
mouse sod1	HEKQDDLKGGNEESTKTGNAGSRLACGVIGIAQXT	156
canine sod1	HEKRDDLKGGDNEESTQTGNAGSRLACGVIGIAQ	153
	* * * * *	

Figure 2. SOD1 protein sequence homology between humans, mice, and dogs (Green et al. 2002)

1991; Green et al. 2002; Tainer et al. 1983). The enzyme functions as a homodimer, and a disulfide bond connects and stabilizes the two subunits (Fridovich 1986; Rakhit and Chakrabartty 2006).

SOD1 is classified as having a Greek key β -barrel structure, named so because diagrams representing the connections between the β -strands are reminiscent of designs found in ancient Greek pottery (Figure 3)(Boissinot et al. 1997). Described as a twisted β -sandwich by Boissinot et. al., each monomer consists of 8 antiparallel β -strands, with 4 strands comprising each half of the sandwich (Figure 3)(Boissinot et al. 1997; Rakhit and Chakrabartty 2006). The 2 halves of the sandwich are covalently bound by 2 Greek key loops found at the ends of the barrel: the longer more complex loop lies between strands 6 and 7, and the shorter loops between strands 3 and 4 (Boissinot et al. 1997).

Disulfide reduced apo-SOD1 polypeptide, the most immature form of SOD1, is properly folded and activated following at least 4 post-translational maturation steps: zinc incorporation, copper incorporation, intramolecular disulfide bond

formation, and dimerization (Furukawa and O'Halloran 2006; Rakhit and Chakrabartty 2006). Also known as Cu,Zn-SOD, each monomer contains one copper (Cu), and one zinc (Zn) ion, which are required for structural stability and enzymatic function (Bannister et al. 1991; Fridovich 1986; Johnson and Giulivi 2005; Rakhit and Chakrabartty 2006). As its name implies, SOD1 catalyzes the dismutation of superoxide anions (O_2^-) (McCord and Fridovich 1969).

Dismutation describes a specific type of redox reaction in which a substrate is reduced and oxidized simultaneously to form 2 different products (McCord and Fridovich 1969). This highly conserved enzyme is capable of converting two molecules of toxic O_2^- into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Figure 3) (McCord and Fridovich 1969; Tainer et al. 1983).

Mutant SOD1 Toxicity in ALS

Mutations in *SOD1* result in the misfolding of the protein (Deng et al. 1993), and altered protein structure has been linked to diminished dismutation activity (Deng et al. 1993; Gurney et al. 1994). In some ALS, however, SOD1 retains almost normal dismutation activity levels (Andersen et al. 1995; Bruijn et al. 1997; Gurney et al. 1994; Wong et al. 1995). Because disease appears to occur independent of normal enzyme activity (Bruijn et al. 1997; Fischer et al. 2012; Gurney et al. 1994; Muller et al. 2006; Wang et al. 2003), it is widely accepted that the role of mutant SOD1 in the ALS disease pathogenesis consists of a gain of function, rather than a loss of function toxicity (Bruijn et al. 1997;

A **B** **C**

Figure 3. Normal SOD1 structure and function (Boissinot et al. 1997). (A) SOD1 has a Greek key topology, named so because diagrams depicting the connections of the 8 antiparallel strands resemble designs found on ancient Greek pottery. (B) Strands are twisted into a “ β -sandwich” with 4 strands comprising each half of the sandwich. The 2 halves are covalently bound by 2 Greek key loops found at the ends of the barrel, and each monomer contains one copper and one zinc involved in maintaining structure and function. (C) Normal enzymatic function involves the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen

Gurney et al. 1994; Ripps et al. 1995; Wong et al. 1995). Of the many SOD1 gain of function hypothesis (see (Boillee et al. 2006a) for review) one hypothesis is that mutant SOD1 is capable of interacting with aberrant substrates generating free radicals (i.e. peroxynitrate, and hydrogen peroxide) and causing oxidative damage to the cell (Beal et al. 1997; Beckman et al. 1993; Ferrante et al. 1997). SOD1 null mice are considered to be a model system for studying oxidative stress in the MU (Fischer et al. 2012). It was reported that these mice developed clinical and histological features consistent with a progressive distal axonopathy (Fischer et al. 2012; Muller et al. 2006), and exhibited evidence of limb muscle weakness and atrophy (Fischer et al. 2012; Flood et al. 1999; Muller et al. 2006; Reaume et al. 1996). They also displayed fiber-typed grouping (Flood et al. 1999; Reaume et al. 1996). SOD1 protein aggregation has also been identified as a potential factor in the ALS disease process (Deng et al. 2006; Prudencio et al. 2009; Wang et al. 2003). Cytoplasmic aggregates containing SOD1 have been identified in spinal cords from *SOD1* associated-ALS patients (Bruijn et al. 1998; Kato et al. 2000; Shibata et al. 1996). Using time-lapsed imaging techniques, Matsumoto et. al. reported a direct association between aggregate formation and cell death in a neural cell line, while cells expressing wild type SOD1 did not develop aggregates nor exhibit premature cell death (Matsumoto et al. 2005). While said study reported a strong correlation between aggregate formation and cell death, this phenomenon may be secondary to disease, and may not represent a direct cause and effect. It is postulated that SOD1 aggregation is caused by aberrant exposure of hydrophobic surfaces, normally buried within the enzyme,

resulting from protein misfolding (Munch and Bertolotti 2010; Zetterstrom et al. 2007). While wild-type SOD1 has been shown to colocalize with mutant SOD1 aggregates (Bruijn et al. 1998; Deng et al. 2006; Prudencio et al. 2010), its contribution in the disease process still is unresolved. In vitro studies showed that overexpression of wild type SOD1 is beneficial in slowing aggregate formation (Prudencio et al. 2009). Likewise studies performed in vivo suggests that wild type SOD1 overexpression does not cause ALS (Gurney et al. 1994; Ripps et al. 1995; Wong et al. 1995), or affect disease onset or duration when over expressed in mutant *SOD1* transgenic mice (Bruijn et al. 1998). Conversely, it has also been reported that overexpression of the wild type protein is indeed capable of inducing the ALS phenotype (Deng et al. 2006; Jaarsma et al. 2000). The pathologic changes of which range from mild histopathologic abnormalities (Dal Canto and Gurney 1995) to significant exacerbation of the disease phenotype (Deng et al. 2006; Jaarsma et al. 2000; Prudencio et al. 2010). Differences in the nature of the *SOD1* mutation and wild type expression levels used in the fore-mentioned studies could be responsible for the discrepancies (Prudencio et al. 2010). Because SOD1 aggregates have been shown to sequester and potentially disrupt proteasome function, one proposed mechanism by which aggregates may cause cytotoxicity is by disruption of the proteasome protein degradation pathway (Matsumoto et al. 2005).

Additionally, the pathogenesis of ALS is considered to be non-cell autonomous, suggesting that mutant *SOD1* exerts its toxic effects on multiple cell types. Restricted mutant SOD1 expression within astrocytes (Gong et al. 2000),

microglia (Beers et al. 2006), or MNs (Lino et al. 2002; Pramatarova et al. 2001) alone is not sufficient to induce an ALS phenotype in rodent models.

Furthermore, mutant MNs within an environment containing wild-type glial cells remain protected against neural degeneration (Beers et al. 2006; Clement et al. 2003). Boillee et. al. reported that mutant *SOD1* expression within MNs dictates disease onset and early disease stage, while expression within glia is responsible for disease progression rates (Boillee et al. 2006b). Also restricted expression of mutant *SOD1* within myofibers is adequate to produce a MN disease phenotype in transgenic mice (Wong and Martin 2010). These studies suggest that development of ALS is dependent on aberrant interactions between multiple cell types and other pathogenic mechanisms. Studies suggest that in ALS MNs become over stimulated when glutamate is not cleared properly from the synaptic cleft by the astrocytic glutamate transporter (GLT-1), resulting in neuronal degeneration (Rothstein et al. 1996). Increased CSF glutamate levels have been identified in ALS patients (Rothstein et al. 1990; Shaw et al. 1995). Likewise decreased GLT-1 levels in transgenic *SOD1* rodents (Bruijn et al. 1997; Howland et al. 2002) and human ALS patients (Rothstein et al. 1995) have been reported.

***SOD1* Transgenic Mouse Models**

Following the discovery that mutations in *SOD1* are associated with familial ALS cases (Rosen et al. 1993), several transgenic *SOD1* rodent models over expressing mutations of *SOD1* have been generated (Bruijn et al. 1997;

Gurney et al. 1994; Jonsson et al. 2006; Ripps et al. 1995; Wong et al. 1995). Common ALS-like phenotypes in these transgenic *SOD1* rodent ALS models include limb weakness and paralysis, and death (Bruijn et al. 1997; Gurney et al. 1994; Turner and Talbot 2008; Wong et al. 1995). Other clinical signs of MN dysfunction include tremor, spasticity, hyperreflexia, positive sharp waves, and neurogenic muscle atrophy (Gurney et al. 1994; Ripps et al. 1995; Wong et al. 1995).

Histopathology of the MU has also been extensively studied in these putative ALS models. These classic pathologies include MN loss, myelinated axon loss, myelin degeneration, atrophic myofibers, and evidence of myofiber type remodeling (Bruijn et al. 1997; Bruijn et al. 1998; Dal Canto and Gurney 1994; Fischer et al. 2004; Guegan et al. 2001; Gurney et al. 1994; Jonsson et al. 2006; Watanabe et al. 2001; Wong et al. 1995). It has been suggested that the progression of these features occur in a “dying back” manner (Dadon-Nachum et al. 2011; Fischer et al. 2004; Frey et al. 2000). Before clinical onset, myelinated axons have been reported to retract from their myofibers, and die back towards the cell body (Fischer et al. 2004). Thus myofibers lose their innervations, resulting in denervation atrophy. As the disease progresses, large myelinated axons preferentially degenerate, and decrease in number. End-stage disease is accompanied by MN degeneration and loss (Fischer et al. 2004).

Transgenic *SOD1* rodent models have also been used to assist in the identification of potential therapeutics for human patients. Unfortunately, numerous clinical trials, initiated in part based on promising preclinical results in

rodent models, have produced a tremendous amount of disappointment in human treatment (Benatar 2007; Morren and Galvez-Jimenez 2012; Scott et al. 2008). Thus there is growing concern about the utilization of transgenic *SOD1* rodent as disease models for human ALS (Benatar 2007; Gordon and Meininger 2011). One suggested limitation of the mouse model is the inapplicable nature of many preclinical study designs (Benatar 2007; Scott et al. 2008; Swash 2007). For example, a large number of rodent studies initiated treatment before the onset of clinical signs, while in human ALS treatment is initiated after clinical onset (Benatar 2007; Gordon and Meininger 2011; Swash 2007). Additionally, the *SOD1*^{G93A} mouse, which over expresses the human *SOD1* mutation (Gurney et al. 1994), has been the standard model used in preclinical studies (Benatar 2007; Scott et al. 2008); however, it has been noted that mice expressing lower copy numbers of the mutant gene more closely embody the human form of the disease (Acevedo-Arozena et al. 2011; Dal Canto and Gurney 1997; Henriques et al. 2010). Overall, there is a critical need for the identification of preclinical ALS models, and the development of applicable study designs that effectively predict therapeutic outcomes in people.

Familial ALS associated with a D90A missense mutation in *SOD1*

The inheritance pattern for most familial ALS is autosomal dominant (Mulder et al. 1986). Interestingly, ALS patients with the *SOD1*^{D90A} mutation have been associated with both an autosomal dominant (Robberecht et al. 1996), and an autosomal recessive (Andersen et al. 1995) inheritance pattern. Al-Chalabi

et. al. suggested these individuals descend from distinct ancestors, such that families with the recessive form also inherited a protective modifier, requiring two copies of the mutant allele to acquire sufficient toxicity to develop disease (Al-Chalabi et al. 1998). With a mean age of onset of 45 years and average disease duration of 13 years, the homozygous recessive *SOD1*^{D90A} patients have a very stereotyped disease progression (Andersen et al. 1996; Andersen et al. 1995). During the “preparetic phase”, individuals experience such symptoms as leg stiffness and weakness, muscular cramps and twitches, and sensory abnormalities, which includes severe leg /back pain and burning sensations, paresthesia, and decreased ability to sense vibration (Andersen et al. 1996). As patients enter the “paretic phase”, they experience progressive asymmetric lower limb paresis, general muscle wasting, fasciculation, and loss of spinal reflexes (Andersen et al. 1996). Roughly 4 years later symptoms appear in upper limbs, succeeded by bulbar symptoms and bladder disturbances (Andersen et al. 1996). As with most ALS, patients typically die of respiratory failure (Andersen et al. 1996). Spinal MNs contain anti-SOD1 positive cytoplasmic aggregates (Forsberg et al. 2010), and muscle biopsies revealed small atrophic fibers and fiber typed grouping (Andersen et al. 1996). Also consistent with the ALS hallmarks, a sural nerve biopsy revealed loss of large caliber axons and myelin pathology (Giannini et al. 2010). We believe that DM closely resembles disease characteristics reported in recessive *SOD1*^{D90A}.

Canine Degenerative Myelopathy (DM)

DM has many features in common with some forms of ALS. DM is a fatal, adult onset, neurodegenerative disease characterized by progressive impairment of sensory and motor functions (Averill 1973). Initial clinical signs are seen in dogs 8 years and older and include general proprioceptive ataxia and spastic weakness in the pelvic limbs. Clinical signs will progress to flaccid tetraplegia and respiratory distress if dogs are not euthanized in early stages of the disease (Awano et al. 2009; Coates et al. 2007; Ogawa et al. 2011; Ogawa et al. 2013). Time of euthanasia is variable, but most pet owners elect to euthanize once the dog can no longer support weight in its pelvic limbs, particularly in larger breed dogs (Table 2) (Coates and Wininger 2010). DM is a prevalent disorder in many breeds including Boxers and Pembroke Welsh Corgis (PWCs). There are no treatments for DM and management of DM affected dogs has been directed toward improving quality of life (Coates and Wininger 2010). While it is known that DM is associated with mutations in the *SOD1* gene, the underlying mechanisms resulting in DM pathology are not well understood (Awano et al. 2009).

For many years DM was believed to only be a disease of the spinal cord (Averill 1973; Griffiths and Duncan 1975). At disease onset, UMN weakness is displayed as increased muscle tone, and intact spinal reflexes. These clinical signs are consistent with disruption of descending motor pathways, and are further supported by the presence of neuronal fiber loss in the descending tracts and subsequent astrocytic gliosis in the spinal cord white matter (Averill 1973; March et al. 2009). Spinal cord histopathology is most severe in the thoracic

Table 2. Clinical grading scale of disease progression in DM-affected dogs

Clinical Grade	Clinical Signs*
1	Paraparesis and General Proprioceptive Ataxia <ul style="list-style-type: none"> • Progressive general proprioceptive ataxia • Asymmetric and spastic paraparesis • Postural reaction deficits in pelvic limb • Intact spinal reflexes (patellar reflex may be decreased)
2	Nonambulatory Paraparesis to Paraplegia <ul style="list-style-type: none"> • Mild to moderate loss of muscle mass in pelvic limbs • Reduced to absent spinal reflexes in pelvic limbs • +/- urinary and fecal incontinence
3	Paraplegia to Thoracic Limb Weakness <ul style="list-style-type: none"> • Signs of thoracic limb weakness • Flaccid paraplegia • Absence of spinal reflexes in pelvic limbs • Severe loss of muscle mass in pelvic limbs • Urinary and fecal incontinence
4	Tetraplegia and Brain Stem Signs <ul style="list-style-type: none"> • Flaccid tetraplegia • Difficulty with swallowing and tongue movements • Absence of spinal reflexes in all limbs • Reduced to absent cutaneous trunci reflex • Generalized and severe loss of muscle mass • Urinary and fecal incontinence

* The presumptive diagnosis of DM for dogs was based on the presence and progression of these signs. Early stages include grades 1-2, and late stages include grades 3-4. (Adapted from (Coates and Wininger 2010; Shelton et al. 2012))

spinal cord early in the disease, but spreads cranially and caudally as the disease progresses (March et al. 2009). It has only recently been recognized that the peripheral nervous system is also affected in DM (Awano et al. 2009; Shelton et al. 2012) If euthanasia is delayed, muscle weakness is exacerbated, widespread loss of muscle mass is observed, and spinal reflexes become reduced to absent. These clinical signs coincide with histopathology of peripheral nerve and hind limb muscles. Shelton et. al. showed that DM is associates with

significant axon loss and myelin changes in the peroneal nerves of affected dogs. This study also revealed evidence of neurogenic muscle atrophy in late stage dogs. Changes in myofiber type distribution were apparent in late stage Boxers; however, PWC hind limb muscle presented more generalized muscle atrophy without type grouping. These findings indicate that the disease processes of DM reach beyond the spinal cord (Awano et al. 2009; Morgan et al. 2013; Shelton et al. 2012). ***The purpose of this study was to characterize the phenotype of a motor unit involved in respiration in DM-affected dogs.*** The distribution of spinal cord, pelvic limb muscle, peripheral nerve lesions and clinical progression in DM are similar to those seen in some forms of *SOD1*-associated ALS (Andersen et al. 1996; Andersen et al. 1995; Awano et al. 2009; Coates and Winer 2010; Giannini et al. 2010; Shelton et al. 2012; Winter et al. 2000). ***Thus we hypothesize that the thoracic MU will exhibit similar histological disease hallmarks as some ALS.*** A thoracic MU was selected for analysis because spinal lesions are more severe in the mid thoracic spinal cord in DM affected dogs (March et al. 2009). Additionally, spinal segments with intact nerve roots are easily accessible from the thoracic cord. Results from this study will increase our understanding of DM and help support whether DM can serve as a reliable ALS disease model. Ultimately by broadening our understanding, these data could contribute to the later development of effective therapeutic interventions for both diseases.

CHAPTER 2

Characterization of Intercostal Muscle Pathology

Abstract

Dogs homozygous for missense mutations in the *SOD1* gene develop a late-onset neuromuscular disorder called degenerative myelopathy (DM) that has many similarities to ALS. Both disorders are characterized by widespread progressive declines in motor functions accompanied by atrophic changes in the descending spinal cord tracts, and some forms of ALS are also associated with *SOD1* mutations. In end-stage ALS, death usually occurs as a result of respiratory failure due to severe functional impairment of respiratory muscles. The mechanisms that lead to this loss of function are not known. Dogs with DM are euthanized at all stages of disease progression providing an opportunity to characterize the onset and progression of any pathological changes in the respiratory muscles that may precede respiratory failure. To characterize such potential disease-related pathology we evaluated intercostal muscles from Boxer and Pembroke Welsh Corgi dogs that were euthanized at various stages of DM disease progression. DM was found to result in intercostal muscle atrophy, fibrosis, increased variability in muscle fiber size and shape, and an alteration in muscle fiber type composition. This pathology was not accompanied by retraction of the motor neuron terminals from the muscle acetylcholine receptor complexes, suggesting that the muscle atrophy did not result from physical denervation. These findings provide a better understanding of the mechanisms

that likely lead to respiratory failure in at least some forms of ALS and will be useful in the development and evaluation of potential therapeutic interventions using the DM model

Introduction

DM is an adult onset neurodegenerative disease characterized by progressive impairment of motor functions (Averill 1973). Initial clinical signs are seen in dogs 8 years or older and include loss of coordination and asymmetric, spastic weakness in the pelvic limbs, which progress to paraplegia within 1 year from clinical onset. If dogs are not euthanized in early disease stages, signs will progress to include flaccid tetraplegia, widespread muscle atrophy and difficulty swallowing (Coates et al. 2007; Coates and Wininger 2010). Dog owners often elect euthanasia when their dogs become paraplegic (Coates and Wininger 2010), but some are not euthanized until more advanced stages of the disease are reached.

DM has many similarities to some forms of human ALS. Like DM, ALS is also an adult onset neurodegenerative disorder characterized by progressive loss of motor functions (Andersen et al. 1996; Charcot J-M 1869). Although causes of most ALS cases are unknown, some sporadic and familial forms have been associated with mutations in *SOD1*, which encodes cytosolic Cu/Zn superoxide dismutase (Rosen et al. 1993). In 2009, we reported that dogs of multiple breeds with a confirmed diagnosis of DM were homozygous for the A allele of a *SOD1* missense mutation, *SOD1*:c.118G>A, which predicts p.E40K

substitution in SOD1 (Awano et al. 2009). Based on clinical signs, genetic testing, and spinal cord histopathology, DM has been found to occur in many breeds, and is especially prevalent in Boxers and Pembroke Welsh Corgis (PWCs) (Coates and Wininger 2010). Despite growing knowledge of genetic associations in ALS, the underlying mechanisms and resulting pathology are still not well understood for either the human or canine disease (Al-Chalabi et al. 2012; Coates and Wininger 2010). A major impediment to elucidating mechanisms underlying disease pathology in ALS is the scarcity of tissues available for study. Tissues that are available from ALS patients are usually obtained postmortem at end-stage disease. Thus, pathologic descriptions of disease progression in ALS patients are few (Fischer et al. 2004). With DM, on the other hand, dogs are euthanized at various disease stages so tissues can be obtained to evaluate progression of disease pathology.

For many years DM was thought to be a disease that involved primarily the ascending and descending tracts of the spinal cord and not the peripheral nervous system (Averill 1973; Griffiths and Duncan 1975). At disease onset, spinal reflexes are consistent with UMN dysfunction. Spinal cord pathology is most evident in the thoracic spinal cord early in the disease, spreads cranially and caudally, and becomes more severe as the disease progresses (Averill 1973; March et al. 2009). More recently, clinical descriptions and histopathology indicate that DM reaches beyond the spinal cord to also involve MUs (MN, axon, and myofiber), at least in the advanced stages of disease (Awano et al. 2009; Shelton et al. 2012). The distribution of lesions and clinical disease progression

in DM are similar to that reported for the UMN-onset ALS, with UMN signs in DM-affected dogs progressing to lower motor neuron (LMN) signs (Brooks et al. 2000; Coates et al. 2007; Coates and Wininger 2010).

To further assess DM as a potential disease model of ALS, studies were conducted to evaluate a thoracic MU in DM-affected dogs. We selected this site because the pathology in the ascending and descending tracts begins in the thoracic region of the spinal cord and because it was anticipated that tissue samples could be obtained from this region that represent all stages of DM disease progression. Thoracic MUs become impaired in ALS causing failure of respiratory muscles (de Carvalho et al. 2010), so evaluation of pathology in this region in DM is likely to be relevant to at least some forms of ALS.

Materials and methods

Tissue collections

Tissues were obtained from companion dogs between 2009 and 2013. Tissues were acquired from 9 Boxers (4 control ages 8 to 13 years; and 5 DM-affected ages 9 to 12 years) and 20 Pembroke Welsh Corgis (PWCs) (6 control, age's 12 to 17 years; and 14 DM-affected, age's 12 to 15 years). Sample collection protocols were approved by the University of Missouri Animal Care and Use Committee. Dogs with a presumptive diagnosis of DM, based on clinical signs of progressive upper motor neuron paresis and general proprioceptive ataxia of the pelvic limbs (Coates and Wininger 2010), were referred to various academic, private specialty or general practices. Dogs were excluded if their

clinical signs were associated with spinal cord compressive lesions detected by magnetic resonance imaging or myelography or if the dog exhibited clinical signs consistent with DM but the histopathology was not typical for a diagnosis of DM [(Coates and Wininger 2010) and Figure 4]. Control tissue was obtained from age and breed matched unaffected dogs euthanized for causes unrelated to DM. Dogs were designated as controls if they had not exhibited clinical signs of DM and histological appearance of the thoracic spinal cord tissue was normal (Figure 4).

Genotypes at canine SOD1:c.118G>A were determined using blood samples from each dog included in the study using a TaqManTM allelic discrimination assay that employed 5'-GTGGGCCTGTTGTGGTATCA-3' with 5'-CAAAGTATGGACGTGGAATCC-3' for the PCR primers and 5'-VIC-CTCGCCTTTAGTCAGC-MGB-3' (A allele) and 5'-FAM-CGCCTTCAGTCAGC-MCB-3' (G allele) for the completing probes (Awano et al. 2009).

When an owner elected to have a dog euthanized, the veterinarian who would perform the necropsy was provided with a kit that included detailed instructions and materials for sample collection, preservation and shipping. Detailed clinical histories were obtained to document the age at onset and progression of clinical signs, neurologic status at the time of euthanasia, and any unrelated health problems. Necropsies were performed within 6 hours after euthanasia. Tissues collected included the T6 spinal cord segment fixed in buffered 10% formalin, the T7 spinal cord segment fixed in 3.5% paraformaldehyde, 0.1% glutaraldehyde, 120 mM sodium cacodylate, 1 mM

CaCl₂, pH 7.4, and sections of intercostal muscle fixed in 2% glutaraldehyde, 1.12% paraformaldehyde, 130 mM sodium cacodylate, 1 mM CaCl₂, pH 7.4 (EM-fix), or in cold 0.17 M sodium cacodylate, pH 7.4. Blood samples for DNA isolation were collected in EDTA vacutainer tubes (Becton Dickinson, catalogue #367899).

Disease status determination

DM-affected dogs were graded based on clinical signs at the time of euthanasia as described in Table 2 (Shelton et al. 2012). T6 spinal cord segments from all dogs included in this study were paraffin embedded and cut into 4 µm thick sections. These sections were stained with H&E, and luxol fast blue-periodic acid Schiff to detect myelin loss. Immunostaining for glial fibrillary acid protein to detect gliosis was performed on dewaxed sections from the same blocks using rabbit anti-GFAP antibody (Dako Z0334; Dako, Carpinteria, CA). Antibody binding was performed as described below, using Nova Red chromogen (Vectorlabs, Burlingame, CA). Diagnosis of DM was histopathologically confirmed by observation of profound axonal loss, myelin loss, and marked astrogliosis in the dorsal portion of the lateral white matter in the thoracic spinal cord (Figure 4) (Averill 1973; March et al. 2009). DM has also been characterized by the presence of cytoplasmic aggregates in spinal cord motor neurons that bind anti-SOD1 antibodies (Awano et al. 2009; Wininger et al. 2011) (Figure 4). T7 spinal cord segments were examined for the presence of these aggregates. To detect SOD1-containing cytoplasmic aggregates, dewaxed

Figure 4. DM affected spinal cords display SOD1 positive aggregates in motor neurons, and marked myelinated axon loss in white matter. Immunohistochemical demonstration of SOD1-containing aggregates in motor neurons in the thoracic spinal cord (A,B). Numerous aggregates stained with an antibody directed against SOD1 were present in ventral horn motor neurons from a DM-affected PWC (B). Similar aggregates were present in all affected dogs of both PCW and Boxer breeds. These inclusions were not observed in thoracic cord motor neurons from unaffected dogs (representative preparation from a control PWC shown in B). Luxol fast blue and periodic acid-Schiff stained thoracic spinal cord from unaffected (C) and grade 4 (D) PWCs. Pallor of the peripheral white matter in (D) corresponds to axonal loss and demyelination (arrows). Higher magnification of thoracic white matter from unaffected (E) and grade 4 (F) PWCs stained with toluidine-blue. Substantial axonal loss with myelin pathology is seen in the PWC with advanced DM. Bars in (A), (C) and (E) indicate magnifications in (B), (D) and (F) respectively

paraffin sections of the T7 spinal cord segment from DM-affected dogs and controls were prepared as described above, incubated overnight at 43°C, then hydrated and steamed at 95°C for 20 minutes in pH 6.0 citrate buffer. After the slides were cooled at room temperature for 20 minutes and rinsed, they were placed in Tris buffer for at least 5 minutes before immunostaining. Next, the slides were treated with 3% H₂O₂ for 15 minutes, washed in buffer, submerged in Protein Blocking solution (Dako) for 5 minutes and drained. They were then incubated in rabbit anti-SOD1 antibody, (Stressgen SOD100) at a 1:800 dilution for 60 minutes at room temperature. Negative controls were treated for 60 minutes with nonimmune rabbit IgG (Sigma, St. Louis, MO) at a 1:1000 dilution in place of the anti-SOD1 antibody. Antibody binding was visualized with the Rabbit Envision+ kit (Dako). The chromogen was Nova Red. Slides were then counterstained in Mayer's Hematoxylin (Newcomer's Supply, Appleton, WI) for 1 minute, dehydrated and cover slipped. Each immunohistochemical specimen was evaluated with and without primary antibody included in the immunostaining procedure to verify the specificity of staining.

Myofiber type grouping and neuromuscular junction analysis

Unfixed intercostal muscles preserved in 0.17 M sodium cacodylate, pH 7.4, were cryo protected by sequentially incubating in 10% sucrose (in 0.17 M sodium cacodylate), 20% sucrose (in 0.17 M sodium cacodylate), and 1:1 20% sucrose: optimum cutting temperature (OCT) solution (OCT, Tissue-Tek, Sakura Finetek, Torrance, CA) for at least 45 minutes each. Samples were then placed

in a plastic mold containing 100% OCT medium, oriented so that muscle fibers could be cut in cross section for myosin heavy chain type 1 (MHC-1) staining, and longitudinally for neuromuscular junction (NMJ) labeling. Molds were incubated on ice for at least 45 minutes, and then transferred quickly to a dry ice block for freezing. Frozen sections were cut at a thickness of 10-12 μ m or 20-30 μ m for MHC-1 and NMJ labeling respectively. Sections were mounted on Superfrost/PlusTM slides (Fisher Scientific #12-550-15). Slides were washed in 1X PBS (Fisher Scientific, Catalog # BP665-1) for 5 minutes before labeling.

Myofiber Type grouping assay.

Endogenous peroxidase was quenched by incubating slides in 3% hydrogen peroxide for 5 minutes at room temperature. Slides were then rinsed in phosphate buffered saline (PBS) (900ml H₂O, 100ml 10x PBS) two times for 5 minutes each, blocked for 1 hour in 5% normal goat serum, incubated with myosin heavy chain 1 antibody (DSHB, Catalog # BA-D5-s) diluted 1:10 overnight at 4⁰C, rinsed in PBS for 5 minutes 3 times, and incubated with goat anti-mouse IgG horseradish peroxidase conjugate (Invitrogen, Catalog # A-10677), diluted 1:10 for 1 hour at room temperature. Slides were then rinsed in PBS 2 times for 5 minutes each, and incubated in diaminobenzidine (DAB, Millipore Catalog number ES005) for 5 minutes at room temperature. With this technique, type 1 fibers stain darkly and type 2 fibers remain unstained. Light microscopy was used to image the entire muscle cross section at a 100x magnification. Each composite image of a muscle section consisted of individual

image frames stitched together. The frames were acquired by moving the specimen past the microscope objective in a raster pattern using an automated stage. Overlapping images of all areas of the section were captured in this manner and stitched into a composite image of the entire section by the Leica software. Proportions of type 1 fibers were calculated from individual frames using ImageJ™ software. For each sample, two frames were randomly selected from each vertical pass of the stage during automated stitching (6-14 frames/sample section depending on size of the cross section). This method of sampling was used to acquire data representative of multiple fascicles within each muscle cross section.

Evaluation of neuromuscular junctions (NMJs).

The intercostal muscles were evaluated for contact between neurons and the muscle acetylcholine receptor (AChR) complexes (Fischer et al. 2004). Cryostat sections of the cacodylate-preserved muscle specimens were incubated with alpha bungarotoxin conjugated to AlexaFluor 594 (Invitrogen, Catalog # B-13423) diluted 1:100 in DI water with 2mM of sodium azide for 45 minutes at room temperature in the dark, then rinsed for 20 minutes in PBS. Slides were then blocked in 5% normal goat serum with 0.9% triton for 1 hour in a humidified chamber, excess blocking reagent was removed, slides were incubated in a humidified chamber overnight in 4°C with anti neurofilament –light (NF-L) antibody (Millipore, Catalog # AB9568) diluted 1:800 in 5% normal goat serum. The sections were then washed 3 times in PBS for 5 minutes each. Secondary

antibody (Invitrogen, AlexaFluor 488 conjugated goat-anti rabbit IgG, Catalog # A-11034), diluted 1:100 in PBS, was applied and the samples were incubated at room temperature in a humidified chamber for 1 hour in the dark. Slides were then washed 3 times in PBS for 5 minutes each, mounted with anti-fade mounding media (Biomedica corp, Gel/Mount aqueous mounting medium with anti-fading agents) and imaged at a 200x magnification. With this technique the muscle AChR's are visualized by the red emission and the axons of the neurons are visualized by the green emission. At least 100 AChR complexes per sample were imaged, and these complexes were examined for the proximity of nerve terminals labeled with the anti-NF-L antibody. Images derived from the red and green channels were layered in PhotoShop, and transparency adjusted to assess the co-localization of labels. Neuromuscular junctions were scored as intact (continuous NF-L label overlapping with bungarotoxin label), partially intact (fragmented NF-L label adjacent to or co-localizing with bungarotoxin label), or absent (bungarotoxin label with no adjacent NF-L label).

Morphometric analysis of myofibers

4 micron thick paraffin cross sections were obtained from EM-fixed intercostal muscle. Sections were cut perpendicular to the long axis of the muscle fibers, and were stained with H&E and Masson trichrome using standard methods. Masson trichrome labeling was used to assess whether fibrosis of the intercostal muscle occurs in DM. Light microscopic images of the sections were obtained at a 200x magnification. FijiTM or PhotoshopTM imaging software were

used to generate a black and white segmented mask (Figure 5. B). In PhotoshopTM, the mask was overlaid on the gray-scaled original image, transparency increased, and manual corrections made to define the boundaries of each fiber (Figure 5.C). Once boundaries are defined, the corrected mask was thresholded in FijiTM, and a particle analysis tool was used to obtain measurements. Morphometric measurements were obtained from at least 100 myofibers per sample and included minor axis length, cross-sectional area, and % of angular fibers (Soraru et al. 2008a; Victor Dubowitz 2007 Brand 1982). The minor axis length is identified as the shortest axis of a best-fit ellipse for each fiber (Figure 5.D). A blind assessment of myofiber angularity was done manually to determine the percent of angulated fibers. Angulated myofibers were defined as those with one or more angles of less than 45 degrees (Inset in Figure 5.C).

Light and fluorescence microscopy

Muscle histological sections were imaged using a Leica DMI 6000B inverted microscope equipped with an automated stage and Leica application suite advanced fluorescenceTM software (LAS-AF) program. For histopathological evaluations, sections were examined using transmitted white light illumination. Images were acquired using a 20 X objective, and individual images were stitched together using the automated stage in combination with the Leica software to create a single high-resolution image of the tissue cross sections (when large areas were to be analyzed for morphological changes). Specimens stained with fluorescent probes were examined with the same microscope using

epi-illumination. To visualize AlexaFluor 594 fluorescence Leica filter cube TX2 ET was utilized (excitation filter band pass [BP] = 560/40; emission filter BP 645/75). To visualize AlexaFluor 488 fluorescence Leica filter cube L5 ET was used (excitation filter BP = 480/40; emission filter BP 527/30).

Statistical analyses

The data for Boxers and PWCs were analyzed independently. Data were analyzed for normality using a Shapiro-Wilk normality test. All data passed the normality test, thus Student's t-test was employed to compare affected and unaffected dogs

Results

SOD1 genotypes

Genotyping results for all dogs are summarized in table 3. All DM-affected PWCs were homozygous for the A allele. Six unaffected PWCs had varying genotypes, including three that were homozygous for the A allele (Table 3). All 5 DM-affected Boxers were homozygous for the A allele. Unaffected Boxers had varying genotypes, including one homozygous for the A allele (Table 3). Of the unaffected PWCs homozygous for the A allele, all were 12 years and older at the time of euthanasia. The unaffected Boxer homozygous for the A allele was 9 years old when euthanized.

Intercostal muscle changes in late-stage DM dogs

Myofibers from unaffected PWCs and Boxers were generally uniform in

Figure 5. Methods for morphometric analysis of myofibers (A) Hematoxylin and eosin stained intercostal muscle cross sections were imaged with light microscopy at a 200x magnification. (B) Fiji™ or Photoshop™ imaging software were used to generate a black and white segmented mask outlining the boundaries of each myofiber. (C) In Photoshop™, the mask was overlaid on the gray-scaled original image, transparency increased, and manual corrections were made to define the boundaries of each fiber. Once boundaries are defined, the corrected mask is thresholded in Fiji™, and a particle analysis tool was used to obtain measurements. Measurements include minor axis length, fiber cross sectional area, and roundness. (D) The minor axis is identified as the shortest axis of a best fit ellipse (Arrow). Pixels of this axis and of the cross sectional areas were converted to μm and μm^2 respectively for each myofiber. The percent of angulated fibers (example labeled with “a” in insert of panel C) were determined manually by a masked evaluator.

Table 3. *SOD1* genotypes

Disease Status - Breed	Number of Dogs of Each <i>SOD1</i> Genotype (age in years)		
	G/G	A/G	A/A
Unaffected PWC	1 (16)	2 (14, 17)	3 (12 to 14)
Affected PWC	0	0	14 (12 to 15)
Unaffected Boxer	1 (8)	2 (12, 13)	1 (9)
Affected Boxer	0	0	5 (9 to 12)

size and shape (Figure 6), with some variability typical of old age (Braund et al. 1982b). For both breeds, myofibers from early disease stage dogs were similar in appearance to those from controls (Figure 6. A). Severe atrophic changes were consistently seen in Grade 4 PWC muscle (Figure 6. C and D). Similar atrophy was occasionally seen in grade 3 PWC, but not in Grade 3 Boxers or in Grade 2 dogs from either breed. No Grade 4 Boxer intercostal muscles were available for this analysis. Collagen labeling was occasionally seen within myofiber fascicles of Grade 4 PWCs (Figure 6. D).

Increased variability in PWC intercostal myofiber size and shape

Individual myofibers were much more variable in size and shape in PWCs with end-stage DM than in age-matched unaffected dogs (Figure 7). Size histograms depict apparent differences in the distribution of the minor axis and area measures of fiber sizes of control and affected PWC myofibers (Figure 7. A and D). When data were divided into early and late disease groups (Figure 7. B and E), there was a larger percentage of myofibers with larger minor axis's in

early stage, and a larger percentage of myofibers with smaller minor axis's in late stage PWC. There was no statistical significance between the mean myofiber minor axes or areas of control and affected PWCs. However, among PWCs there was a significantly larger range of myofiber sizes in the muscles from DM dogs compared to those from unaffected dogs (minor axis length range; control=

Figure 6. Severe pathology in intercostal muscle of late stage DM dogs
Intercostal muscles were stained with H&E (A and C), and Masson trichrome (B and D), to visualize general morphology of myofibers and presence of fibrotic tissue. (A and B) Myofibers from a control PWC display uniform size and shape with no fibrosis seen between fibers. (C) Myofibers from a grade 4 PWC showing variability in size and shape with hypertrophy (long arrow) and atrophy (short arrow) fibers. (D) Late-stage PWC displaying fibrosis (blue stain).

28.2 μm , affected = 43.4 μm : $p < 0.001$, area range; control = 1,737 μm^2 affected = 2,8491 μm^2 : $p = 0.039$) (Figure 7. C and F). The percentage of angulated myofibers was on average larger in grade 4 PWC muscle than in unaffected PWCs (Control = $7.7\% \pm 3.5\%$ ($n = 6$ dogs), affected = $14.5\% \pm 6.9\%$ ($n = 4$ dogs); $p = 0.068$). These data support the abnormal deviation from uniform fiber sizes seen in figure 6, and coincide with the observation of abundant small atrophic fibers and large hypertrophic fibers in Grade 4 intercostal muscle.

Among Boxers, there were no differences in fiber size distributions between affected and unaffected dogs (Figure 8). However there was a peak in the medium-size range of both minor axis length and area parameters in early disease samples that was not present in the normal or late stage dogs (Figure 8 B and E).

Acetylcholine receptor (AChR) complexes remain innervated in DM affected intercostal muscle

Because atrophic myofibers are a prominent feature in late stage PWC intercostal muscle, we evaluated muscle AChR complexes to determine if they maintained close proximity with nerve terminals (Figure 9 A-C). No differences were seen between the percentages of intact, partially intact, or absent axons from affected and control dogs (Figure 9. D).

End stage intercostal muscles display shift to type 1 myofiber predominance

Unaffected and early stage Boxer and PWC muscles retained a normal checkerboard pattern of type 1 and type 2 fibers distributions (Figure 10. A). Late

Figure 7. Morphometric data reveal increased variability of intercostal myofiber sizes in DM-affected PWC. (A, D) Size distribution graphs of myofibers from affected PWCs show changes in the distribution of fibers sizes (minor axis, and cross sectional area respectively). (B, E) Distribution graph of affected PWCs separated into early (n = 3) and late (n=9) stages. Early stage myofiber distributions were shifted towards larger size classes, while more small fibers were seen in the late stage distribution. (C, F) No significant difference was seen between the mean minor axis lengths or cross sectional area of unaffected and affected dogs; however the range of fiber sizes were significantly increased in affected PWCs (* p<0.05).

Figure 8. Boxer morphometric data reveal no significant differences in intercostal myofiber sizes. (A, D) Size distributions of minor axis length and cross-sectional area of myofibers from unaffected and affected samples. (B, E) When affected dog data is separated into early and late stage (early: n=2, Late: n=2), early disease samples exhibit a peak in the medium size range. (C, F) No significant differences were detected in mean minor axis length or range between control and affected groups.

stage PWC samples displayed a dramatic shift to a predominance of type 1 fibers (Figure 10. B). The percentages of type 1 myofibers in an unaffected and grade 3 PWC samples were similar (Unaffected: 32%, grade 3, 39% and 38%). In two grade 4 PWCs, the percentage of myofibers that were of type 1 were 67%

Figure 9. Acetylcholine receptor (AChR) complexes remain innervated in DM-affected intercostal muscle. Thoracic intercostal muscle from one unaffected and six affected dogs were stained with alpha bungarotoxin to label AChR's (red), and anti-NF-L to label axon terminals (green). At least 100 complexes were imaged from each sample. Axons were scored based on proximity to AChR complexes as intact (A), partially intact (B), or absent (C). (D) Bar graph showing the percentage of receptors from each sample that were intact, partially intact, or absent. Insert in A is a higher magnification showing contact between the nerve terminal and the receptor complex. A-C are representative images of observations from control and affected samples.

and 76%. Two Boxers, grade 2 and 3 had moderately higher proportions of type 1 myofibers (59% and 65%, respectively) than the one unaffected Boxer (49%). The control and Grade 3 Boxers had higher proportions of type 1 fibers compared to the PWCs with the same disease statuses. Only a limited number of samples suitable for these analyses were available because MHC-1 immunolabeling was hindered by formalin fixation.

Figure 10. Changes in fiber type patterns in intercostal muscle of late stage DM dogs. Intercostal muscles were stained with anti-myosin heavy chain-1 to visualize the proportions of fiber types. (A) Unaffected PWC with normal checkerboard pattern of type 1 and 2 fibers. (B) Grade 4 PWC with a high predominance of type 1 fibers. Bar in (A) represents magnification in both images. (C) Bar graph displaying % of type 1 myofibers in PWC (left) and Boxer (right) thoracic intercostal muscle. PWC graph correlates with observations, with a larger percentage of type 1 fibers in grade 4 dogs (control n = 1, affected n = 4). (Boxers: control n = 1, affected n = 2).

Discussion

Muscle atrophy is a classic and progressive feature in ALS patients and has also been reported in *SOD1* transgenic mouse models (Andersen et al. 1996; Charcot J-M 1869; Gurney et al. 1994; Wong et al. 1995). Our data indicate that this atrophy is recapitulated in the intercostal muscles in canine DM. The DM-related changes in muscle morphology are consistent of those associated with denervation atrophy, suggesting neurogenic pathology due to altered motor neuron input to the muscle (Shelton et al. 2012). However there did not appear to be loss of physical contact of the motor nerve terminals with the intercostal muscle fibers. Among dogs with DM at the stages evaluated in this study, the disease is not accompanied by a loss of motor neurons from the thoracic cord or decreases in the numbers of motor axons in thoracic cord motor nerve (March et al. 2009). Therefore, if muscle atrophy was secondary to altered neural input, it appears that the cause must be primarily impaired motor neuron function without gross structural changes in the motor nerve terminals, axons or cell bodies. The finding that the neuromuscular junctions remain physically intact differs from findings in some *SOD1* transgenic mouse models in which loss of contact between motor neuron terminal processes and muscle acetyl choline receptor complexes has been reported (Dadon-Nachum et al. 2011; Fischer et al. 2013; Fischer et al. 2004; Frey et al. 2000; Schaefer et al. 2005).

In a recent study, electrodiagnostic evaluations of dogs with DM indicated functional impairment of pelvic limb muscles in early disease stages when dogs

become nonambulatory, whereas histopathological evidence of denervation was not apparent until advanced disease stages (Shelton et al. 2012). Similar findings have been described in some ALS and Charcot-Marie-Tooth mouse models where abnormal motor function and muscle pathology existed without significant physical evidence of denervation (Lobsiger et al. 2005; Shen et al. 2011). In ALS patients, electrodiagnostic evaluation of diaphragm and intercostal muscles revealed fibrillation sharp waves, indicative of denervation, which correlated with respiratory muscle weakness (de Carvalho et al. 2010). Whether there is a similar physical preservation of the neuromuscular junctions in human ALS intercostal muscles remains to be determined. This will be important to evaluate with respect to further assessing both DM and SOD1 transgenic mice as models for ALS.

In comparing pathological changes associated with DM or ALS, it is important to note that similar changes may occur at different stages of the disease in different muscle groups and in the nerves associated with these muscles. Weakness and atrophy of limb muscles are relatively early to mid-stage signs of both ALS and DM, whereas respiratory impairment is a relatively late manifestation of these diseases. Therefore, the intercostal muscles evaluated in this study most likely represent the earlier phase of the degenerative process and therefore provide information on a different stage of the pathogenesis than the hind limb muscles obtained at the same disease stages (Shelton et al. 2012). Likewise, the fibular nerve pathology reported in DM (Shelton et al. 2012) may be

more advanced at the same disease stage than any pathology that might be present in nerves associated with the intercostal muscles.

It has been widely reported that neurogenic atrophy in muscle from ALS patients is accompanied by fiber-type grouping, postulated to occur when denervated myofibers are reinnervated by collateral sprouts from neighboring axons (Andersen et al. 1996; Fischer et al. 2004; Schaefer et al. 2005; Soraru et al. 2008a). On the other hand, it has also been reported that muscle biopsies from ALS patients exhibit muscle fiber atrophy but no fiber type grouping (Baloh et al. 2007). The reason for this apparent discrepancy is not apparent from the data available, but ALS actually encompasses a spectrum of disorders, and it is possible that fiber type grouping occurs in some forms of the disease and not others (Soraru et al. 2008a). The pronounced increase in the ratio of type 1 to type 2 fibers seen in end stage PWCs, relative to that in a normal PWC, has also been reported in hind limb muscle from an ALS mouse model with a low copy number of a mutant SOD1 transgene (Acevedo-Arozena et al. 2011). This change could result from fiber-type remodeling. Alternatively, DM may result in a preferential loss of type 2 fibers. In ALS mouse models, myofibers expressing the type 1 myosin isoform are more resistant to disease (Acevedo-Arozena et al. 2011; Frey et al. 2000; Hegedus et al. 2007). This could also be the case for the intercostal muscles in DM and in at least some forms of ALS.

Although the fiber type composition was evaluated in a limited number of dogs in the present study, it appears that there may be significant breed differences in distribution of fiber types in the intercostal muscles (Armstrong et

al. 1982). Thus, for evaluation of disease progression in DM, it appears that data from different breeds should not be pooled. Because PWCs tend to be euthanized at all stages of the disease process whereas very few Boxers are maintained until disease end stage, it would be more practical to focus future studies on the PWC breed as a disease model.

In DM histopathology, both in the intercostal muscles and in pelvic limb muscles, occurs late in the disease process (Shelton et al. 2012), suggesting that DM may be a very useful model for studying the mechanisms underlying the development of muscle pathology in ALS. Tissue samples available from ALS patients, particularly nervous tissues, have primarily been collected postmortem from patients who died as a consequence of the disease and not prior to the development of advanced histopathology. On the other hand, tissue samples can be collected from PWCs at all stages of DM disease progression. Larger breed (Boxers) dogs are usually euthanized when nonambulatory paraparesis develops so tissues representing late stage disease are difficult to obtain from this or other larger breed with DM. Functional and biochemical analyses of early stage muscle and nerve is likely to provide insight into the process that culminates in the types of late-stage muscle histopathology characteristic of both DM and ALS.

In this study, we found that not all dogs homozygous for the SOD1 mutation develop DM and none of the dogs that were heterozygous for this mutation developed the disease. In a cohort of 239 dogs of multiple breeds that were homozygous for the A allele and over 9 years of age, 25 had not exhibited

signs of DM (unpublished data). This is consistent with a previous report that this SOD1 mutation in DM is associated with an incompletely penetrant autosomal recessive mode of inheritance (Awano et al. 2009). Similarly, it has also been reported that the D90A- SOD1 mutation in ALS patients can be inherited in an autosomal recessive and incompletely penetrant pattern (Al-Chalabi et al. 1998; Andersen et al. 1996; Andersen et al. 1995; Khoris et al. 2000). Patients with the adult onset recessive form of ALS are speculated to have also inherited a protective modifier, tightly linked with SOD1, from a common ancestor (Al-Chalabi et al. 1998). This hypothetical modifier is proposed to decrease the toxicity of the mutant allele so that two copies are required to produce an ALS phenotype. The existence of a hypothetical modifier similar to that which has been proposed for ALS could also explain why some dogs that are homozygous for the SOD1:c.118G>A mutation fail to develop DM, even at advanced ages. Determining why some dogs that are homozygous for the SOD1 mutation fail to develop DM may provide clues on how to prevent or delay the development and progression of DM in dogs and ALS in people with predisposing SOD1 mutations.

Overall these studies indicate that DM may be a very useful model in elucidating the mechanisms underlying neuromuscular pathology in at least some forms of ALS. The muscle pathology in the canine disease recapitulates that which occurs in the human disorder. The ability to obtain tissues from affected dogs in the early stages of the disease process will enable characterization of the pathogenesis of disease progression that is not possible

with ALS. DM is also likely to be a good disease model for evaluating potential therapeutic interventions for some forms of ALS

CHAPTER 3

Characterization of Thoracic Motor and Sensory Neurons and Spinal Nerve Roots

Abstract

Canine Degenerative Myelopathy (DM) is a progressive adult-onset multisystem degenerative disease with many features in common with amyotrophic lateral sclerosis (ALS). As with some forms of ALS, DM is associated with mutations in superoxide dismutase 1 (*SOD1*). Clinical signs include general proprioceptive ataxia and spastic upper motor neuron paresis in pelvic limbs, which progress to flaccid tetraplegia and dysphagia. The purpose of this study was to characterize DM as a potential disease model for ALS. We previously reported that intercostal muscle atrophy develops in dogs with advanced stage DM. To determine if other components of the thoracic motor unit (MU) also demonstrated morphological changes consistent with dysfunction, histopathologic and morphometric analyses were conducted on thoracic spinal motor neurons (MN) and dorsal root ganglia (DRG), and in motor and sensory nerve root axons from DM-affected Boxers and Pembroke Welsh Corgis (PWCs). No alterations in MNs, or motor root axons were observed in either breed. However, advanced stage PWCs exhibited significant losses of sensory root axons, and numerous DRG sensory neurons displayed evidence of degeneration. These results indicate that intercostal muscle atrophy in DM is not preceded by physical loss of the motor neurons innervating these muscles, or of

their axons. Axonal loss in thoracic sensory roots and sensory nerve death suggest sensory involvement may play an important role in DM disease progression. Further analysis of the mechanisms responsible for these morphological findings would aid in the development of therapeutic intervention for DM and some forms of ALS

Introduction

Canine degenerative myelopathy (DM) is a hereditary neurodegenerative disorder with onset of clinical signs occurring in dogs 8 years or older (Averill 1973). Clinical signs appear in pelvic limbs as spastic upper motor neuron (UMN) paresis and general proprioceptive ataxia, which progresses to flaccid tetraplegia and dysphagia if euthanasia is delayed (Coates and Wininger 2010). Like some forms of familial amyotrophic lateral sclerosis (ALS) DM is associated with mutations in *SOD1* (Andersen et al. 1996; Coates and Wininger 2010); (Morgan et al. 2013). DM occurs in many breeds and is typically accompanied by the *SOD1* missense mutation *SOD1*:c.118G>A, which predicts a p.E40K substitution (Awano et al. 2009). The clinical signs, disease progression, and muscle pathology in DM are similar to those seen in some forms of human *SOD1*-ALS. Thus it appears that DM may potentially be a good disease model for some types of ALS.

In ALS patients who receive extensive palliative care, death usually results from failure of respiratory muscle function, typically within 3-5 years after clinical onset. While many ALS patients are medically managed and survive for relatively

long periods in severely debilitated states with respiratory and feeding assistance, DM affected dogs are usually euthanized at earlier disease stages often due to ambulatory impairments. However, some DM-affected dogs are maintained until respiratory dysfunction develops (Ogawa et al. 2011; Ogawa et al. 2013). Therefore, both ALS and DM are characterized by the occurrence and progression of UMN and lower motor neuron (LMN) signs, which ultimately result in flaccid tetraplegia and respiratory failure (Brooks et al. 2000; Coates and Wininger 2010; Ogawa et al. 2011; Ogawa et al. 2013) (Table 2).

Histopathological similarities between DM and ALS include myelinated axon loss and gliosis within the spinal cord, myelinated axon loss in peripheral nerves and muscle atrophy. Accumulations of cytoplasmic aggregates containing SOD1 within motor neurons (MNs) also occurs in DM and some forms of ALS (Andersen et al. 1996; Awano et al. 2009; Charcot J-M 1869; Forsberg et al. 2010; Hirano 1991; Shelton et al. 2012; Sobue et al. 1987) (Morgan et al. 2013; (Averill 1973; Giannini et al. 2010)). Although ALS is generally considered primarily a disease of the motor system, there is growing evidence indicating sensory involvement (di Trapani et al. 1986; Dyck et al. 1975; Guo et al. 2009; Heads et al. 1991; Isaacs et al. 2007; Kawamura et al. 1981; Mulder et al. 1983; Pughahl et al. 2007). Sensory deficits are also characteristic features of DM (Averill 1973).

Although the degenerative changes in the spinal cord neuronal fibers have been known for some time (Griffiths and Duncan 1975); (Averill 1973), LMN involvement in DM is a relatively new finding (Awano et al. 2009; Coates et al.

2007; Shelton et al. 2012). We recently reported that intercostal and pelvic limb muscles from Pembroke Welsh Corgis (PWCs) at late stage disease exhibited marked pathological changes consistent with denervation atrophy (Morgan et al. 2013; Shelton et al. 2012). Also, thoracic MNs that innervate the intercostal muscles contain cytoplasmic aggregates that stain with an anti-SOD1 antibody (Awano et al. 2009; Morgan et al. 2013). The degenerative changes in the intercostal muscles suggest that thoracic motor units are dysfunctional in DM, and may result from impaired MN input to these muscles. In most forms of ALS loss of spinal MNs and axonal degeneration are considered disease hallmarks (Hirano 1991; Sobue et al. 1981), and loss of MN input appears to play an important role in the pathology that develops in the associated muscles. To assess whether the thoracic muscle atrophy in DM is also associated with MN defects, morphometric analyses were performed on thoracic MNs and motor axons from DM-affected dogs and age-matched control dogs. In addition, evaluations were performed to determine whether DM is accompanied by alterations in the thoracic sensory nerves and their axons

Materials and methods

Tissue collections from dogs

Tissues were obtained from companion dogs between 2009 and 2013. Samples were acquired from 14 Boxers (6 control ages 8 to 12 years; and 9 DM-affected ages 10 to 12 years) and 21 Pembroke Welsh Corgis (PWCs) (7 control, ages 13 to 17 years; and 15 DM-affected, ages 12 to 15 years) (Table

4). Dogs were diagnosed with presumptive DM at academic, private specialty or general practices based on clinical presentation and progression of upper motor neuron and lower motor neuron signs, and general proprioceptive ataxia that began in the pelvic limbs (Coates and Wininger 2010). Histopathological evaluations of the thoracic spinal cords were performed on all of these dogs. Only those dogs that had exhibited clear clinical signs of DM, and thoracic spinal cord lesions characteristic of this disease (Averill 1973) were designated as DM-affected. Control tissue was obtained from age and breed matched unaffected dogs euthanized for causes unrelated to DM. Dogs were designated as controls if they had not exhibited clinical signs of DM and histological appearance of the thoracic spinal cord tissue was normal. Dogs that exhibited clinical signs similar to those of DM were excluded if their clinical signs were associated with spinal cord compressive lesions detected by magnetic resonance imaging or myelography or if the thoracic spinal cord histopathology was not typical for a diagnosis of DM [(Coates and Wininger 2010).

Genotypes at canine SOD1:c.118G>A were determined for each dog included in the study using a TaqMan™ allelic discrimination assay that employed 5'-GTGGGCCTGTTGTGGTATCA-3' with 5'-CAAAGTATGGACGTGGAATCC-3' for the PCR primers and 5'-VIC-CTCGCCTTTAGTCAGC-MGB-3' (A allele) and 5'-FAM-CGCCTTCAGTCAGC-MCB-3' (G allele) for the completing probes (Awano et al. 2009). The classification of the dogs by disease status and genotype is shown in Table 5

When an owner elected to have a dog euthanized, the veterinarian who would perform the necropsy was provided with a kit that included detailed instructions and materials for sample collection, preservation and shipping. Detailed clinical histories were obtained to document the age at onset and progression of clinical signs, neurologic status at the time of euthanasia, and any unrelated health problems. Necropsies were performed within 6 hours after euthanasia. Protocols for tissue collection were approved by the University of Missouri Animal Care and Use Committee. Tissues collected included thoracic spinal cord segment 6 (T6) fixed in buffered 10% formalin, the T7 segment fixed in 3.5% paraformaldehyde with 0.1% glutaraldehyde and 120 mM sodium cacodylate, 1 mM CaCl₂, pH 7.4 (Immuno-fixed), and the T8 segment (with ventral and dorsal roots, and dorsal root ganglion intact) fixed in 2% glutaraldehyde, 1.12% paraformaldehyde, 130 mM sodium cacodylate, 1 mM CaCl₂, pH 7.4 (electron microscopy [EM]-fix). Blood samples for DNA isolation were collected in EDTA vacutainer tubes (Becton Dickinson, catalogue #367899).

Disease status determinations

Confirmation of disease status for each dog was performed as previously described(see chapter 2). Briefly, disease stages at time of euthanasia were determined using a clinical grading scale (Table 2. Clinical grading scale of disease progression in DM-affected dogs) (Shelton et al. 2012). DM diagnoses were confirmed histopathologically by assessing the T6 cord segment for

evidence of myelinated axon loss and pronounced astrogliosis in the dorsal the typical histopathology were presumed to have another cause for the myelopathy and excluded from the study. T7 spinal cord segments were examined for the presence of SOD1 immunoreactive aggregates within ventral portion of the lateral funiculus (Averill 1973; March et al. 2009). Dogs that had exhibited clinical signs of DM but did not show horn motor neurons (Awano et al. 2009). Dogs that had not exhibited any clinical signs of DM prior to euthanasia and whose thoracic spinal cords were histologically normal were used as controls. Tables 4 and 5 provide a summary of the disease status of the dogs employed in this study.

Table 4. Summary of the number of dogs within each disease grade

Numbers of dogs in each grade					
Breed	Control	Grade 1	Grade 2	Grade 3	Grade 4
PWC	7	0	5	4	6
Boxer	6	3	3	2	1

*See Table 2 for definitions of disease grades

Table 5. *SOD1* genotypes

Disease Status & Breed	Number of Dogs of Each <i>SOD1</i> Genotype (age in years)		
	G/G	A/G	A/A
Unaffected PWC	1 (16yrs)	4 (13-17yrs)	2 (13-14yrs)
Affected PWC	0	0	15 (12-15yrs)
Unaffected Boxer	0	4 (8-12 yrs)	2 (9yrs)
Affected Boxer	0	0	9 (10-12 yrs)

Spinal cord motor neuron cell body counts

An immuno-fixed T7 spinal cord segment from each dog was embedded in paraffin. From each paraffin-embedded sample, 4µm sections (each separated by at least 100µm) were placed on positive charged slides (1 section per slide), and left flat on a 430C slide warmer overnight. The slide-mounted sections were steam-treated in a decloaking chamber (Biocare Medical, Concord, CA.) at 980C for 30 minutes in citrate target retrieval solution (Dako, Carpinteria, CA.), cooled at room temperature for 10 minutes, rinsed with distilled water, then placed on IntelliPATH FLX autostainer for staining. Slides were treated with 3% H2O2 for 15 minutes, washed in buffer, treated with Sniper block (Biocare Medical, Concord, CA.) for 20 minutes. They were then incubated in primary antibody goat anti-choline acetyl transferase (ChAT) (Millipore AB144P;1:200) for 60 minutes. Control sections were treated with nonimmune rabbit IgG (Sigma, St. Louis, MO.) at a 1:1000 dilution for 60 minutes in place of the anti-ChAT antibody. The detection system used was rabbit anti-goat horse radish peroxidase (HRP) kit (PK6105; Biocare Medical, Concord, CA.) with 30 minute incubations in link and label steps followed by a tris buffer rinse. The sections were then incubated in romulin red (Biocare Medical, Concord, CA.) for 10 minutes. Slides were counterstained in hematoxylin (Biocare Medical, Concord CA.) at a 1:10 dilution for 5 minutes then followed with a tris-buffered rinse, dehydrated, coverslipped, and then imaged with light microscopy at 200x magnification. The numbers of ChAT-positive cell bodies in Rexed lamina 9 per section were determined by counting the cells in a sufficient number of sections from each spinal cord sample

such that a minimum of 100 cells per dog were counted. Using Image J™ software, cross-sectional area (CSA) was determined for all ChAT-positive cells in Rexed lamina 9. CSA and diameter of motor neurons with visible nucleoli were also determined

Morphometric and pathologic analyses of nerve roots

Approximately 3 mm long segments of ventral and dorsal roots were dissected from the EM-fixed T8 cord segments. The samples were post-fixed with 2% osmium tetroxide and subjected to extended microwave infiltration and embedded in epon araldite resin. After resin embedding, blocks were polymerized at 60°C for 48 hours, and nerve cross-sections were cut at a thickness of 0.4µm. The sections were stained in 2% P-phenylene diamine (PPD) solution (MP Biomedicals, LLC, catalog # 151830) (1g PPD in 50ml of 50% EtOH) made up 5 days to 2 weeks prior to use. Sections were incubated in filtered PPD solution for 15 minutes, rinsed 1-3 times in ultra-pure H₂O, covered with Permunt mounting medium and coverslipped. High resolution images of each stained nerve cross section were obtained using a Leica DMI 6000B inverted scope equipped with a programmed stage and Leica application suite advanced fluorescence software™ (LAS-AF). Using automated stage controls and image acquisition individual images totaling the entire area of each section were obtained using a 40X objective and stitched together by the LAS-AF software to create a single high-resolution image of the tissue cross section. The sharpness and contrast of the composite images were optimized using

Photoshop™ software. The resulting images were then analyzed using MetaMorph® image analysis software to obtain the following morphometric data for each nerve: total myelinated axon number, endoneurial area, endoneurial area occupied by axons, percent axon occupancy (percentage of endoneurial space occupied by myelinated axons), axon density, and axon size distribution. The image analysis procedure is summarized in figure. 11. PPD and toluidine blue stained sections of the motor roots were examined for histopathological abnormalities by an experienced pathologist (GDS) in a masked manner. Fibers with inappropriately thin myelin sheaths relative to axon diameter, myelin splitting and ballooning, axonal degeneration and number of probable regenerating clusters (defined as two or more closely apposed myelinated fibers) were assessed in each nerve specimen. As previously described (Shelton et al. 2012), only those fibers with clear, faintly staining space evident between separated myelin with an asymmetrical profile are considered to have splitting and ballooning, while profiles containing paranodal regions or Schmidt-Lanterman clefts were not counted

Statistical analyses

Data for each breed was evaluated independently. Each data set was evaluated for normality using the Shapiro-Wilk normality test. For data that were

Figure 11. Procedure for obtaining morphometric measurements in T8 motor and sensory root cross sections. (A) Resin embedded nerves were sectioned and stained with P-phenylene diamine (PPD), imaged using light microscopy, and composite images of the entire nerve cross-sections were constructed. (B) Magnified image of area outlined in (A). (C) Images were sharpened using Photoshop™ tools. (D) Imaris® software was used to invert the image, and remove background noise. (E) Multicolored image produced by the automated count tool in MetaMorph® software. Colors indicate what software distinguishes as individual objects. Note that several adjacent axons are being mistaken for a single object. (F) Manual corrections were made in Photoshop by overlaying, and increasing the transparency of, the multicolored image over the sharpened light micrograph. (G) Multicolored images after manual corrections. (H) Corrected multicolored image of entire nerve root cross-section. (Illustration represents a T8 motor root cross section).

normally distributed Student's t-test was employed to determine whether significant changes exist between affected and unaffected groups. When data were analyzed using disease stage (early and late) as a variable, data were subjected to analysis of variance followed by Holm-Sidak analyses to compare individual groups. Data that were not normally distributed were analyzed using the Mann-Whitney ranked sum test

Results

T7 motor neuron density and morphology

DM did not result in significant changes in mean motor neuron densities or mean cell body cross-sectional areas of the T7 spinal cord segment for either breed compared to controls, even when data for all affected dogs within each breed were pooled (Figure 12.C), or when control samples were compared only to late stage DM samples (data not shown). In some affected and unaffected samples of both breeds motor neuron morphological abnormalities (i.e. vacuoles, eccentric nuclei, and shrinkage) were occasionally seen, with no apparent association with disease status (Figure 12. B).

No disease related changes were seen in ventral root motor axons

Among Boxers and PWCs, DM was not associated with significant alterations in the motor root features that were analyzed relative to age matched unaffected dogs, even in dogs in the most advanced stages of clinical disease (Tables 6 and 7). Total numbers of axons in the motor roots were similar

Cross Sectional Area (μm^2)					
PWC	# of Dogs	Mean	Standard Deviation	Range	Density (# of cells/section)
Unaffected	5	724.21 $\pm$ 145.11	424.30 $\pm$ 41.17	1,920.82 $\pm$ 320.10	13 $\pm$ 1
Affected	8	838.48 $\pm$ 200.03	516.79 $\pm$ 72.92	2,154.93 $\pm$ 381.23	15 $\pm$ 4
P-Value		0.29	0.02 *	0.27	0.17

Boxer					
Unaffected	# of Dogs	Mean	Standard Deviation	Range	Density (# of cells/section)
Unaffected	3	467.81 $\pm$ 226.64	308.23 $\pm$ 151.36	1,257.43 $\pm$ 590.91	18 $\pm$ 4
Affected	7	438.68 $\pm$ 46.20	305.29 $\pm$ 33.42	1,240.24 $\pm$ 169.38	22 $\pm$ 4
P-Value		1	0.83	0.83	0.30

Figure 12. Thoracic motor neuron morphometric measurements. (A, B) T7 spinal cord section from an unaffected 9 year old Boxer stained with ChAT. (B). Higher magnification of highlighted box within Rexed lamina 9 in A. This image is a representation of the normal motor neuron morphology seen in unaffected and affected dogs of both breeds. (C) Morphometric measurements obtained from PWC (top) and Boxers (Bottom). Pooled data indicate no difference in the motor neuron density between unaffected and affected samples of either breed (*Affected boxers: one grade 1, three grade 2, one grade 3, and two grade 4 dogs; Affected PWC: two grade 2, two grade 3, and four grade 4 dogs*). Likewise no differences were seen in the mean soma cross sectional area (CSA) or size range. In PWC's a significant difference was seen in the CSA standard deviation, suggesting more variability in soma area in affected dogs ($p=0.02$). One Way ANOVA indicated the difference in standard deviation is attributed to a significant difference between unaffected and late stage (grade 3-4) PWC samples ($p=0.04$).

between affected and normal dogs within each breed. Among both normal and affected dogs, the Boxers consistently had larger numbers of axons in the thoracic motor roots than did the PWCs (Tables 6 and 7). Additionally, when data were separated into early and late stage groups within each breed, no significant differences were detected in any morphometric measurements compared to control unaffected motor roots (data not shown). Size distribution histograms reveal a bimodal distribution of axon diameters typical of thoracic ventral roots (Figure 12). The smaller caliber peak (≤ 5 microns) represents axons of autonomic neurons from the intermediolateral horn (Sobue et al. 1981). The larger caliber peak (>5 microns) likely represents alpha motor axons (Braund et al. 1982a). There were no differences in the relative distribution of axons

Table 6. PWC T8 motor root morphometric measurement results

PWC T8 Motor Roots	Unaffected	Affected	P value
Number of dogs	5	8	
Average count of axons	3,676 $\pm$ 262	3,863 $\pm$ 691	0.57
Average endoneural CSA (μm^2)	308,081.54 $\pm$ 39,460.49	297,826.80 $\pm$ 55,818.72	0.72
Average area occupied by axons (μm^2)	191,114.56 $\pm$ 35,453	179,168.71 $\pm$ 24,679.03	0.48
% of endoneural area occupied by axons	61.7% $\pm$ 6.1%	60.7 $\pm$ 5.8%	0.78
Average axon density count/mm ²)	12,165.08 $\pm$ 2,131	13,400.49 $\pm$ 3,874.93	0.53

* 5 unaffected and 8 affected (four grade 2, one grade 3, and three grade 4) dogs

Table 7. Boxer T8 motor root morphometric measurement results

Boxer T8 Motor Roots	Unaffected	Affected	P value
Number of dogs	5	8	
Average count of axons	4,697 ± 827	4,140 ± 1,104	0.35
Average endoneural CSA (μm^2)	443,654.10 ± 94,990.21	464,871.30 ± 124,963.56	0.75
Average area occupied by axons (μm^2)	267,532.55 ± 69,695.82	269,769.55 ± 62,814.24	0.95
% of endoneural area occupied by axons	60.3 % ± 8.5%	59.1% ± 9.8%	0.83
Average axon density (count/ mm^2)	10,850.21 ± 2,368.04	9,039.09 ± 2,143.59	0.22

*5 unaffected and 8 affected (two grade 1, three grade 2, one grade 3, and two grade 4) dogs

between these two size classes between samples from unaffected and affected dogs (Figure 13). Histopathologic changes including myelin pathology, axonal degeneration, and presumptive regenerative clusters were present in control and affected samples from both breeds (Table. 8). Since no apparent association with disease was noted, these changes likely represent features of normal aging or may also include postmortem artifacts.

Significant axonal loss in T7 dorsal roots of DM-affected Pembroke Welsh Corgis

Late stage DM (grades 3 and 4) was accompanied by an average 23% reduction in the number of T7 dorsal root axons of PWC's (unaffected: 3,261 ± 354, affected: 2,498 ± 324, $p = 0.003$) (Figure 14 and 15); however, no differences were seen in the other morphometric measurements including average

Table 8. Histopathological assessment motor root myelinated axons

Grade	Breed	Splitting, ballooning, and myelin thickness	Axonal degeneration	Regenerative clusters
0	Boxer	++, numerous thin	++	possible
0	Boxer	+, splitting, thin, regional	0	possible rare
0	Boxer	+++ thin	++	possible yes
0	Boxer	++, numerous thin	+	few
0	Boxer	0	0	0
1.5	Boxer	numerous thin	++	possible yes
1.5	Boxer	numerous thin	++	possible yes
2	Boxer	numerous thin	+	few
2	Boxer	few split, few thin	0- +	0
2.5	Boxer	few split, few thin	0- +	few
3	Boxer	few split, + thin	0	0
4	Boxer	numerous thin	0	possible yes
4	Boxer	several thin, regional	0	few
0	PWC	few thin	0	few
0	PWC	++, numerous thin	++	possible yes
0	PWC	rare thin	0	few
0	PWC	++, numerous thin	0	few
0	PWC	+, moderate thin	+++	few
2	PWC	+, numerous thin	0	few
2	PWC	+, numerous thin	0	possible yes
2	PWC	+	0	uninterruptable
2	PWC	+, ++	+	0
4	PWC	+, ++	0	0

* Mild pathology = +. Moderate = ++, Severe = +++

A **B**

PWC	Unaffected	Affected	P value
% of axons $\leq 5 \mu\text{m}$	62.30%	64.47%	0.87
% of axons $> 5 \mu\text{m}$	37.70%	32.53%	0.52
Average # of axons $\leq 5 \mu\text{m}$	2,086 $\pm$ 268	2,427 $\pm$ 593	0.25
Average # of axons $> 5 \mu\text{m}$	1,587 $\pm$ 253	1,430 $\pm$ 151	0.18

C **D**

Boxers	Unaffected	Affected	P value
% of axons $\leq 5 \mu\text{m}$	63.32 %	59.08%	0.89
% of axons $> 5 \mu\text{m}$	36.68%	40.92%	0.52
Average # of axons $\leq 5 \mu\text{m}$	2,954 $\pm$ 671	2,445 $\pm$ 807	0.30
Average # of axons $> 5 \mu\text{m}$	1,765 $\pm$ 283	1,708 $\pm$ 376	0.79

Figure 13. Motor root myelinated axon diameter distribution from PWC's and Boxers. Motor root myelinated axon diameter distribution from PWC's (A,B) and Boxers (C,D). Histograms show a bimodal size distribution with one peak representing small caliber axons, and one peak representing large caliber axons (A, C). For both breeds, no obvious differences could be seen between large caliber axons from control and affected motor roots, while the small caliber peak was more variable. (B, D) For both breeds, no statistical difference was seen in the percentage or average numbers of axons $\leq 5 \mu\text{m}$. Data represented in this figure were obtained from the same samples depicted in tables 6 and 7.

endoneurial cross sectional area, average area occupied by axons, % of endoneurial area occupied by axons, and average axon density (Table 9). The axons in the sensory roots also exhibited a bimodal distribution in axon diameters (Figure 15). The disease-related decrease in axon number in sensory roots was seen in both large ($>5\mu\text{m}$) and small ($\leq 5 \mu\text{m}$) size classes (Figure 15). Morphological features indicative of myelin pathology and axonal degeneration were not disease-associated (Table 10). Among the affected PWCs, the dorsal root ganglia (GRGs) had large numbers of cells with dark condensed cytoplasm and pyknotic nuclei (Fig. 14D) which were not seen in DRGs from age-matched unaffected dogs (Fig.1 4C).

Discussion/Conclusion

Despite substantial atrophic changes that occur in the intercostal muscles in late stage DM (Morgan et al. 2013), no alterations in the numbers of thoracic lower motor neurons nor their axons were observed in dogs with this disorder. Likewise, we previously reported that there was no evidence of denervation of the intercostal muscle acetyl choline receptors in DM (Morgan et al. 2013), unlike

the denervation reported in some putative mouse models of ALS (Dadon-Nachum et al. 2011; Fischer et al. 2004; Schaefer et al. 2005). These data indicate that intercostal muscle atrophy in DM is not secondary to loss of physical contact with motor neurons, yet may be secondary to functional defects in the motor neurons that are not reflected in obvious structural changes in the nerves. In a recent study by Ogawa et al. that included Japanese DM-affected PWCs exhibiting respiratory signs, loss of thoracic motor neurons was observed

Figure 14. PWC sensory root and dorsal root ganglia histopathology. Histological sections of T8 sensory roots (A and B) and dorsal root ganglia (DRG) (C and D) in PWC samples. EM-fixed sensory roots and DRG from control (A,C) and grade 4 (B,D) PWC samples were embedded in resin and stained with PPD and toluidine blue respectively. Compared to controls (A), sensory roots from grade 4 PWC (B) exhibited decreased myelinated axon density. DRG neurons from grade 4 PWC (D) contained numerous cells with condensed cytoplasm (arrowhead) and severely pycnotic nuclei (arrow) that were not present in the DRG neurons from age-matched control dogs (arrowhead and arrow in C denote cytoplasm and nucleus of normal neuron for comparison).

Table 9. PWC morphometric sensory root data

PWC T8 Sensory Roots	Unaffected	Affected	P value
number of dogs	6	6	
Average count of axons	3,261 ± 354	2,498 ± 324	0.003*
Average endoneural CSA (μm^2)	299,035.80 ± 51,135.46	287,306.10 ± 104,376.02	0.81
Average area occupied by axons (μm^2)	168,275.15 ± 50,113.15	131,682.17 ± 28,538.90	0.15
% of endoneural area occupied by axons	56.9% ± 15.1%	48.2 ± 9.5%	0.26
Average axon density (count/mm ²)	11,078.84 ± 1,663.80	9,606.45 ± 3,192.25	0.34

*affected samples: two grade 3, four grade 4

(Ogawa et al. 2013). In Japan, DM- affected dogs are often maintained even after the onset of respiratory distress (Ogawa et al. 2011; Ogawa et al. 2013), while in other countries dogs with DM are usually euthanized when they become nonambulatory in the pelvic limbs (Coates and Wininger 2010). Based on a comparison of our findings with those from the study of the PWCs from Japan, it appears that motor neuron loss may be a feature of DM that only becomes apparent in the terminal stage of disease.

In late stage DM intercostal muscles display a marked increase in type-1 myofibers(Chapter 2), similar to that observed in a *SOD1* transgenic mouse model with a low copy number of the mutant allele (Acevedo-Arozena et al. 2011). Because myosin isoform expression is dictated by the motor neuron (Buller et al. 1960), this change suggests pathology within the motor neuron. DM

thoracic motor neurons contain accumulations of insoluble SOD1-containing cytoplasmic aggregates, which increase over disease duration (Crisp et al. 2013) (Awano et al. 2009). Moreover, Ogawa et. al., showed that astrocytes in thoracic and cervical gray matter have reduced expression of glutamate transporter 1 (GLT-1) (Ogawa et al. 2013), and increased iNOS labeling (Ogawa et al. 2011) which may contribute to dysfunction of the associated motor neurons. Therefore, rather than analyzing the physical preservation of the motor unit

Figure 15. PWC sensory root myelinated axon diameter distribution. (A) Histogram shows a bimodal size distribution with one peak representing small caliber axons, and one peak representing large caliber axons. (B) There was a statistically significant difference in the average number of axons $\leq 5 \mu\text{m}$ ($p=0.03$) and $>5 \mu\text{m}$ ($p=0.03$). Data represented in this figure were obtained from the same samples depicted in table 9

structures, future studies should be targeted to obtain a better understanding of

Table 10. Histopathological assessment sensory root myelinated axons

Grade	Breed	Splitting, ballooning, and myelin thickness	Axonal degeneration	Regenerative clusters
0	PWC	+ thin	+	++ sprouts
0	PWC	+ thin, demyelinated, partially myelinated	0	0
0	PWC	++ split, ballooning	0	0 (aging?)
0	PWC	+ split, ++ thin	+	0
0	PWC	+ split, ++ thin	+	0
0	PWC	+ ballooning, ++ thin	0	0
2	PWC	++ thin	0	+++ possible
3	PWC	+ thin	0	0
3	PWC	+ thin	0	0
4	PWC	rare split, ballooning	0	0
4	PWC	+ thin, + ballooning	0	0
4	PWC	+++ thin	0	0
4	PWC	+ thin, ballooning	0	0

* Mild pathology = +. Moderate = ++, Severe = +++

the molecular dysfunction within spinal motor neurons and non-neural supporting cells of DM dogs.

This study was consistent with a previous suggestion that DM dogs exhibited severe degeneration in lumbar sensory roots and minor chromatolysis in DRG, with relative sparing of lumbar motor roots report (Griffiths and Duncan, 1975). Although clinical signs of DM and ALS are primarily associated with motor dysfunction, sensory deficits also are known to occur as part of the clinical spectrum of both diseases (Andersen et al. 1996; Andersen et al. 1995; Averill 1973; Coates and Wininger 2010); (Dyck et al. 1975; Isaacs et al. 2007; Mulder et al. 1983; Pugdahl et al. 2007). For example, sensory symptoms in patients with ALS have been reported to include numbness in the legs and a burning

sensation in the feet, decreased light touch and pinprick sensation, reduced vibration sense, and reduced laryngeal sensation. Additionally, sensory fiber loss has been reported in sural nerves (di Trapani et al. 1986; Giannini et al. 2010; Heads et al. 1991; Isaacs et al. 2007), and dorsal roots (Kawamura et al. 1981) in familial and sporadic ALS patients and in transgenic SOD1 rodent models (Guo et al. 2009). Within the disease spectrum of ALS, cases with sensory involvement may represent a variant of the disease or a continuum of the multisystemic neurodegeneration (Heads et al. 1991). Degenerative myelopathy may serve as a disease model for a subset of ALS patients. Our data indicating that significant loss of sensory neurons precedes any physical loss of motor neurons in the thoracic spinal cord suggests that more thorough investigation of the potential role of sensory pathology in ALS is warranted.

Every dog in which DM has been confirmed, both on the basis of clinical signs and the presence of characteristic pathological lesions in the thoracic spinal cord, has been homozygous for the SOD1:c.118G>A mutation, so there is little doubt that this mutation is closely associated with the disease. However, among the PWCs examined in this study, affected dogs were euthanized as a consequence of the disease as early as 12 years of age, whereas two of the seven unaffected PWCs were homozygous for this mutation and had not exhibited any of the clinical signs of DM or any spinal cord pathology by 13 to 14 years of age. This indicates that there are other factors that determine whether a genetically predisposed dog develops DM or at least the age at which the disease develops. An understanding of the factors that modify the age of onset

and rate of disease progression in DM may be useful in developing effective treatments for ALS.

DM as a disease model may serve as a valuable tool to investigate disease mechanisms, therapeutic strategies and disease markers for ALS. It is important to continue to thoroughly characterize DM, and develop outcome measures to provide biomarkers for reliable assessments of the efficacy of potential therapeutic interventions

CHAPTER 4

General Discussion

In this study, we have shown that intercostal muscle atrophy occurs in late stage DM without physical evidence of MN or motor axon degeneration. Also there was no loss of physical synaptic connectivity with nicotinic acetylcholine muscle receptor complexes. Although the MN soma appeared to be physically intact, the pattern of muscle pathology was consistent with defects in neuronal input. For example, intercostal myofiber variability seen in grade 4 samples was consistent with denervation atrophy, suggesting primary dysfunction within the MN. The change observed in fiber type distribution in grade 4 PWC samples may have also been indicative of changes in neuronal input.

Intercostal muscle pathology seen in this study had several similarities and difference compared to that seen in hind limb muscle of DM affected dogs. Early stage Boxer intercostal and hind limb muscles had minimal pathologic changes, while late stage Boxer hind limb muscle revealed extensive fiber typed grouping and marked myofiber size variability (Shelton et al. 2012). Unfortunately, because Boxers were generally euthanized at earlier disease grades, late stage intercostal muscle available for the current study was limited. Therefore it is unknown whether intercostal muscle pathology correlated with that seen in hind limb muscle in DM affected Boxers. Grade 4 PWC hind limb muscle histopathology did not exhibit fiber type grouping and had an appearance which resembled a mixture of denervation and accelerated sarcopenia (Shelton et al.

2012), differing from the denervation atrophy and fiber type distribution changes observed in this study. These results indicate that disease related histopathological changes may differ between breeds and MUs (i.e. thoracic MU vs. lumbar MU).

The increase in type 1 myofiber predominance could have been from substantial fiber-typed grouping, in which a large portion of myofibers were reinnervated with neurons dictating a type 1 phenotype. Type 1 myofibers have been shown to be more resistant to the ALS disease process than type 2 and/or axons innervating type 1 fibers have a better capacity to sprout in response to disease (Acevedo-Arozena et al. 2011; Frey et al. 2000; Hegedus et al. 2007; Schaefer et al. 2005). In 1994, Gurney et. al. reported that distal axonal sprouting likely compensated for muscle death until late in the disease course in the *SOD1*^{G93A} mouse (Gurney et al. 1994). In a muscle displaying scarcely denervated endplates, 90% of myofibers in the gluteus muscle were innervated by two surviving axons of the inferior gluteal nerve (Gurney et al. 1994). In low copy number *SOD1*^{G93A} mice, Acevedo-Arozena et. al. also reported substantial increases in type 1 myofibers of the extensor digitorum longus muscle, suggested by the authors to be a result of fiber-typed grouping (Acevedo-Arozena et al. 2011). Therefore reinnervation by axonal sprouts from neurons dictating a type 1 myofiber phenotype could explain the lack of denervated muscle receptors and the increase in type 1 myofibers observed in grade 4 PWC intercostal muscles.

Alternatively, because we did not observe any loss of or alterations in diameter distributions of myelinated axons, MU structures may have lost functional synaptic connections. Studies in transgenic mutant *SOD1* mice, utilizing inhibition of apoptosis (Dewil et al. 2007; Gould et al. 2006) or administration of histone deacetylase inhibitors (Rouaux et al. 2007), showed that MN death is dissociated from motor dysfunction. In these studies, MN somas and proximal axons were protected from degeneration; however, progressive functional decline persisted. Similar results were reported in which preservation of MUs, including neuromuscular junctions, occurred in transgenic mouse models for *SOD1*-ALS and Charcot-Marie-Tooth disease 2E (Lobsiger et al. 2005; Shen et al. 2011). In these reports, preservation of MU structures also did not prevent functional decline. MU function may also be compromised by alterations in synaptic input from cortical and brain stem neurons (Sasaki and Maruyama 1994; Sunico et al. 2011). MN and motor root axon loss were recently reported in terminal DM affected PWC displaying respiratory impairment (Ogawa et al. 2013). Therefore, it was possible that physical loss of MU structures only occurred in terminal DM disease stages; however, functional deficiency preceded physical loss.

Muscle atrophy associated with *SOD1* mutations may be partially due to direct effects of the mutations on the muscle rather than secondary to alterations in MN input (Marcuzzo et al. 2011; Wong and Martin 2010; Zhou et al. 2010). Supporting this concept, restricted expression of mutant *SOD1* within muscle of transgenic mice was sufficient to generate a MN disease phenotype comparable

to ALS (Wong and Martin 2010). In a similar study Dobrowolny and colleagues demonstrated that myofiber restricted mutant *SOD1* expression in transgenic mice was sufficient to cause severe muscle atrophy, decreased muscle strength, and sarcomere and sarcotubuloar disorganization (Dobrowolny et al. 2008). These authors also reported an alteration in mitochondrial distribution associated with a shift in myofiber metabolism from glycolytic to oxidative in hind limb muscle (Dobrowolny et al. 2008). In primary LMN diseases such as spinal and bulbar muscular atrophy (SBMA), histological features consistent with myopathic diseases have been identified (Onesto et al. 2011). For example, In addition to characteristic neurogenic atrophy, skeletal muscle pathology in patients and mouse models with SBMA also revealed features of primary muscle disease including splitting and necrotic fibers (Amato et al. 1993; Harding et al. 1982; Mariotti et al. 2000; Sobue et al. 1989; Soraru et al. 2008b). Therefore the muscle pathology observed in this study could partially be associated with primary abnormalities within myofibers.

Results from this study indicated significant pathology within DRG neurons and sensory root axons. In the initial description of DM, Griffiths and Duncan reported histopathologic changes in these sensory structures in lumbar spinal regions (Griffiths and Duncan 1975). Sensory root axons displayed evidence of degeneration and DRG neurons exhibited features of cell death, with relative sparing of lumbar motor roots and MNs (Griffiths and Duncan 1975). The present study complimented these findings with more detailed methodology and emphasized the significant impact of DM on sensory pathways. A reduction in

DRG neurons and sensory root axons from the L5 spinal segment in ALS patients has been reported (Kawamura et al. 1981). Similar results were also seen in the *SOD1*^{G93A} mouse with progressive dorsal root axon loss and DRG degeneration (Guo et al. 2009). The function of MNs innervating intercostal muscles was partially dependent on a feedback loop that involved the sensory neurons from the same cord segment. It was possible that the pathology exhibited by thoracic sensory neurons resulted in secondary abnormal function of the MNs they innervated, and consequently was involved in the intercostal muscle atrophy observed (Ausborn et al. 2009; Mentis et al. 2011).

The purpose of this study was to further characterize DM through MU analysis. However, on a broader scope our goal was to assist in establishing whether DM could serve as a reliable ALS disease model. In addition to what has already been described in DM, the results from this study suggested that DM is a good disease model for some forms of ALS. DM is mainly a recessively inherited, disease associated with mutations in *SOD1* (Awano et al. 2009). There was disruption of motor and sensory pathways initiating in the lower limbs (Averill 1973; Griffiths and Duncan 1975). Disease progression was very stereotyped and predictable (see Table 11)(Coates and Winger 2010). Although the fate of the majority of ALS patients is the same (i.e. muscle paralysis, and premature death), the disease progression and onset is heterogeneous; therefore, not all forms of ALS can be compared to canine DM. However, *SOD1*^{D90A} was one form of ALS that shared many of these characteristics with DM (Table.11) (Andersen et al. 1996; Andersen et al. 1995). ALS is a devastating disease and there are no

effective treatments for any form of ALS. Because DM resembles the recessive *SOD1*^{D90A} form of ALS, we believe that it will serve as a disease model in our ultimate goal of developing treatments for ALS patients and DM affected dogs.

Compared to transgenic mutant *SOD1* mice, DM affected dogs in this study were not genetically altered and had a naturally occurring mutation in the *SOD1* gene (Table. 11) (Awano et al. 2009). Because the *SOD1* mutation was not overexpressed, as with the most commonly used transgenic mutant *SOD1* mouse model (Gurney et al. 1994), the disease progression in DM affected dogs was more comparable to human ALS (Coates and Wininger 2010). Another important defining feature of DM affected dogs was that these companion animals shared the same environment as humans, and some environmental factors have been considered risk factors for ALS development (Ahmed and Wicklund 2011; Fang et al. 2010; Garruto et al. 1989; Roelofs-Iverson et al. 1984; Spencer et al. 1986). While our knowledge about the pathogenic mechanisms of ALS was derived from transgenic rodent studies, there is growing concern about the preclinical application of these rodents in ALS (Benatar 2007; Gordon and Meininger 2011). Canine DM as another tool will serve as a suitable bridge for this translational gap.

Table 11. Comparative summary of human ALS and ALS disease models

	Genetics	Clinical onset	Disease progression	Disease Duration	Motor Unit histopathology	Sensory defects	Bladder dysfunction
ALS (all forms)	<ul style="list-style-type: none"> 90% sporadic 	variable	variable	variable	<ul style="list-style-type: none"> MN loss motor axon loss neurogenic atrophy fiber typed grouping in some forms 	yes, rare	no
SOD1 FALS	<ul style="list-style-type: none"> 2% of all ALS >160 mutations Mostly dominant 	variable	variable	variable	<ul style="list-style-type: none"> MN loss motor axon loss neurogenic atrophy fiber typed grouping[◇] 	yes [◇]	no
SOD1-D90A human FALS	<ul style="list-style-type: none"> recessive and dominant 	stereotyped	stereotyped	protracted	<ul style="list-style-type: none"> MN loss motor axon loss?? neurogenic atrophy fiber typed grouping 	yes	yes
High copy # transgenic G93A SOD1 mice	<ul style="list-style-type: none"> overexpression of SOD1 mutation 	stereotyped	stereotyped	rapid	<ul style="list-style-type: none"> significant MN death late NMJ disruption early neurogenic atrophy fiber typed grouping dying back pathology 	yes	no
SOD1 canine DM	<ul style="list-style-type: none"> naturally occurring mutation mostly recessive 	stereotyped	stereotyped	protracted	<ul style="list-style-type: none"> MN and motor axon death in terminal stages no NMJ disruption neurogenic atrophy fiber typed grouping?? 	yes	yes

*High copy number SOD1^{G93A} transgenic mouse is the most commonly used model in ALS preclinical studies

◇D90A SOD1 FALS

?? Undetermined

CHAPTER 5

Future Directions

The work conducted in this study contributed to the understanding of canine DM by broadening our general knowledge of the MU pathology involved in the DM disease progression. Additionally, results from this investigation have laid a foundation for future studies that are needed to further characterize canine DM.

As mentioned previously, MN and motor axon loss in DM may be features of terminal disease stage in the mid thoracic spinal cord. It is unlikely that we would be able to characterize MUs at terminal disease stages in this country due to animal welfare concerns. However analyzing MUs from other spinal cord segments could give us a more complete picture of the temporal degeneration of other MUs in DM. Ravits et. al. reported that in human ALS patients MN loss is focally more severe at the site of clinical onset (Ravits et al. 2007). Clinical signs begin in the pelvic limbs and later progress to involve the thoracic limbs and bulbar functions in DM-affected dogs (Awano et al. 2009; Coates and Wininger 2010). Therefore, within the same dog it is possible for us to evaluate MUs at various time points in the degenerative process. In future studies, morphometric methods, similar to the ones used in the present study, could be applied on spinal cord segments collected from the lumbar and cervical intumescences and brain stem motor nuclei.

Along with our finding of denervation atrophy in DM-affected PWC

intercostal muscles, respiratory dysfunction in DM was a novel finding (Ogawa et al. 2011; Ogawa et al. 2013). DM-affected PWCs in Japan have been reported to display clinical evidence of respiratory muscle impairment at terminal disease stages (Ogawa et al. 2011; Ogawa et al. 2013). In those studies, clinical evaluations of these dogs were not extensively described (Ogawa et al. 2011; Ogawa et al. 2013). Because ALS patients die as a result of weakness and paralysis of respiratory muscles, including the intercostal muscles (de Carvalho et al. 2010), it will be important to further investigate the possibility that DM would

Figure 16. Tidal breathing flow-volume loop. Loops are plotted as volume (mL) vs flow (L/sec). Normal loops resemble a “D” shape. Inspiration begins on the right side and continues clockwise as inspiration ends, and expiration begins (arrows). Changes in loop shape may be indicative of respiratory abnormalities.

give rise to the same fate in dogs. Therefore further studies should be conducted to evaluate the onset and early progression of respiratory muscle abnormalities in DM-affected dogs.

Pulmonary function testing is regularly used as a biomarker in the ALS field to monitor the progression of respiratory deficiencies (Brinkmann et al. 1997; Czaplinski et al. 2006). Common methods employed in human medicine require voluntary patient cooperation. For instance forced vital capacity (FVC) evaluates the amount of air a patient can move out of the thoracic cavity in a forced maneuver (Brinkmann et al. 1997; Czaplinski et al. 2006). Because dogs are not able to comply with such requests, pulmonary function techniques have been adapted for use in small animals. One example is the assessment of tidal breathing flow-volume loops (TBFVL) (Figure 16), which quantitatively measures airflow patterns at rest (Rozanski and Hoffman 1999). In an awake animal, a tight-fitting mask with a pneumotachograph (apparatus for recording rate of air flow) is placed over the muzzle. Loops are then plotted as volume (mL), and flow (L/sec) on the x, and y axis respectively (Miller 2007; Rozanski and Hoffman 1999). Normal loop appearance resembles a “D” (see figure above). Inspiration begins on the right hand side and the loop continues clockwise as inspiration ends, and expiration begins (Rozanski and Hoffman 1999). Calculated loop indices (i.e. tidal volume [mL], inspiratory time [msec], expiratory time, peak inspiratory flow [mL/sec], peak expiratory flow, etc) have been identified for dogs (Rozanski and Hoffman 1999), and loops can be evaluated for change in shape and corresponding parameters to identify respiratory abnormalities (Miller 2007;

Rozanski and Hoffman 1999). This may be one technique that could be applied to DM dogs to monitor changes in respiratory pattern caused by muscle weakness over the disease course.

Another future direction would be to further assess pathologies at the molecular/cellular level occurring within DM motor neurons. Previous reports suggest that the *SOD1* mutation associated with DM has a gain of function toxicity, in which SOD1 protein aggregation may be a key factor in the disease process (Crisp et al. 2013). The gain of function toxicity is supported by the normal enzymatic activity level retained by the mutant enzyme. In DM (Crisp et al. 2013) and in *SOD1*-ALS mice with low mutant copy numbers (Acevedo-Arozena et al. 2011; Dal Canto and Gurney 1997; Henriques et al. 2010), gradual accumulation of insoluble aggregates over time has been reported. Although this association may not establish cause and effect, increase in aggregate accumulation tightly linked to disease severity may indicate a toxic association between disease progression and protein aggregation (Crisp et al. 2013; Matsumoto et al. 2005). This has been proposed in ALS (Bruijn et al. 1998; Deng et al. 2006; Matsumoto et al. 2005; Prudencio et al. 2009). One potential way to evaluate aggregate involvement in DM is to prevent or slow the formation of aggregates and assess the change in aggregate formation with disease severity. Antisense oligonucleotide therapy involves the use of a ~20 nucleotide sequence construct which binds via Watson-Crick hybridization to its complementary mRNA sequence, causing the targeted mRNA to be degraded by RNase H (Smith et al. 2006). Administered intrathecally, antisense oligonucleotides

generated against SOD1 mRNA have been shown to be widely distributed in brain and spinal cord regions of mutant *SOD1*- ALS transgenic rats and non-human primates (Smith et al. 2006). The construct penetrated into the nervous tissue, localized in spinal motor neurons and astrocytes, reduced SOD1 expression, delayed disease progression, and extended survival (Smith et al. 2006). Recently a phase 1 placebo –controlled clinical trial reported that antisense oligonucleotides were safe for application in human *SOD1*-ALS patients, (Miller et al. 2013) indicating that this approach may serve as a promising treatment for some forms of ALS. Plans are underway to utilize this technology in DM-affected dogs, and because misfolded SOD1 in DM is prone to aggregate (Crisp et al. 2013), decreasing protein expression, by degrading SOD1 mRNA, may in turn modulate aggregate formation. Filter trap assays have been used on mutant *SOD1* rodent nervous tissue (Hishikawa et al. 2003; Wang et al. 2003), and in vitro using cells expressing human (Matsumoto et al. 2005) and canine *SOD1* mutations (Crisp et al. 2013) to assess aggregate formation. Additionally, Crisp et. al. employed western blotting analysis on spinal cord homogenates from DM affected dogs, and reported an increase in detergent-insoluble mutant SOD1 which corresponded with disease severity (Crisp et al. 2013). Similar protocols could be used for assessment of antisense oligonucleotide influence on SOD1 aggregation in DM-affected dogs. Results of which would be compared to disease severity to evaluate if (1) the treatment is capable of reducing SOD1 aggregates, and (2) to determine if that reduction affects disease phenotype.

General proprioceptive ataxia is an early clinical sign of DM (Averill 1973); however, as the disease progresses it is difficult to clinically separate the motor and sensory functional loss. Therefore, histological assessment may be a valuable tool in evaluating sensory involvement in DM.

Sensory axon abnormalities have been reported in several ALS cases. Sural nerve biopsies (a purely sensory nerve) harvested from ALS patients early in the disease with no electrophysiological evidence of sensory involvement revealed significant morphometric changes in sural nerve studies compared to normal controls, suggesting early sensory axonal atrophy in ALS that may be masked by motor degeneration (Hays et al. 1991). However axonal density was unchanged in this study, suggesting that axon drop out may occur later in the disease progression (Hays et al. 1991). Additionally, Giannini et al. reported a progressive decrease in sensory action potential (SAP) amplitudes and sensory conduction velocities (SCV) in sural nerves from a FALS *SOD1*^{D90A} patient (Andersen et al. 1995), indicating axonal loss and myelin degeneration. This observation was confirmed by a sural nerve biopsy revealing marked axonal degeneration and dropout (Giannini et al. 2010). Progressive decreases in SAPs and SCVs have also been reported in sporadic ALS cases (Isaacs et al. 2007; Pugdahl et al. 2007). Interestingly, one patient was reported to have absent SAPs, although axonal loss in a sural nerve biopsy was mild with moderate myelin pathology (Isaacs et al. 2007). Taken together these results support the idea that ALS is a multisystem disease affecting the motor and sensory system. These data also suggest that sural nerve axonal loss may be a feature of late

disease (Heads et al. 1991), and functional loss may precede physical axon loss (Isaacs et al. 2007). To expand our histological assessment of the sensory system in DM, sural nerve biopsies could be harvest from early and late stage dogs for morphometric evaluation of myelinated axons. Additionally, electrodiagnostic evaluation of sural nerves could also be employed, to monitor the progression of sensory deficits. Also, to further evaluate dorsal root and DRG involvement in the DM disease progression, our analysis should be extended to other spinal segments, and should encompass early and late disease grades.

Peripheral nerve axon loss in DM has been reported (Shelton et al. 2012). However, the nerve analyzed was a mixed nerve (common peroneal); consequently, it is still to be determined if the loss observed was due to loss of motor, sensory or both axon types. To complement the work done by Shelton et. al., peroneal nerves could be subjected to double immunofluorescence labeling using antibodies against ChAT and NF-L using a protocol previously described (Gay et al. 2012). With this method all axons would be labeled with anti NF-L, and only motor axons would be identified with ChAT labeling. Imaging analysis software could then be used on captured images to obtain counts of the total number of NF-L positive axons vs ChAT positive axons, allowing us to determine what proportion of the peroneal nerve axon population is motor vs. sensory fibers. One limitation with this method would be control over antigen availability. For instance, within a given motor axon segment ChAT antigen availability could be limited resulting in a false negative. One way to control for this would be to stain multiple sections per sample and/or to identify sensory axon specific

antibodies. This analysis would need to be conducted on early and late stage DM samples to evaluate the progression of axon loss.

Summary

This study was the first report of respiratory muscle histopathology in DM, complementing recent findings that DM ultimately resulted in respiratory impairment (Ogawa et al. 2011; Ogawa et al. 2013). Our data also supported recent findings of LMN involvement in DM (Awano et al. 2009; Shelton et al. 2012). Although no morphological defects were observed in thoracic spinal MN somas, the muscle pathology observed in the intercostal muscles was suggestive of MU pathology. Our findings of substantial sensory pathology in dorsal roots and dorsal root ganglia emphasized that DM was a multisystem disease encompassing the degeneration of both motor and sensory pathways. The results from this study significantly contribute to the study of canine DM and provide further insight into the similarities between DM and ALS patients with autosomal recessive *SOD1*^{D90A} ALS. Overall, we believe that canine DM is a reliable disease model for human ALS, and further studies using this model will contribute to our understanding of both diseases and development of effective treatments.

REFERENCES

- Acevedo-Arozena A, Kalmar B, Essa S, Ricketts T, Joyce P, Kent R, Rowe C, Parker A, Gray A, Hafezparast M, Thorpe JR, Greensmith L, Fisher EM. 2011. A comprehensive assessment of the SOD1G93A low-copy transgenic mouse, which models human amyotrophic lateral sclerosis. *Disease models & mechanisms* 4(5):686-700.
- Ahmed A, Wicklund MP. 2011. Amyotrophic lateral sclerosis: what role does environment play? *Neurol Clin* 29(3):689-711.
- Al-Chalabi A, Andersen PM, Chioza B, Shaw C, Sham PC, Robberecht W, Matthijs G, Camu W, Marklund SL, Forsgren L, Rouleau G, Laing NG, Hurse PV, Siddique T, Leigh PN, Powell JF. 1998. Recessive amyotrophic lateral sclerosis families with the D90A SOD1 mutation share a common founder: evidence for a linked protective factor. *Hum Mol Genet* 7(13):2045-2050.
- Al-Chalabi A, Jones A, Troakes C, King A, Al-Sarraj S, van den Berg LH. 2012. The genetics and neuropathology of amyotrophic lateral sclerosis. *Acta Neuropathol* 124(3):339-352.
- Amato AA, Prior TW, Barohn RJ, Snyder P, Papp A, Mendell JR. 1993. Kennedy's disease: a clinicopathologic correlation with mutations in the androgen receptor gene. *Neurology* 43(4):791-794.
- Andersen PM, Forsgren L, Binzer M, Nilsson P, Ala-Hurula V, Keranen ML, Bergmark L, Saarinen A, Haltia T, Tarvainen I, Kinnunen E, Udd B, Marklund SL. 1996. Autosomal recessive adult-onset amyotrophic lateral sclerosis associated with homozygosity for Asp90Ala CuZn-superoxide dismutase mutation. A clinical and genealogical study of 36 patients. *Brain* 119 (Pt 4):1153-1172.
- Andersen PM, Nilsson P, Ala-Hurula V, Keranen ML, Tarvainen I, Haltia T, Nilsson L, Binzer M, Forsgren L, Marklund SL. 1995. Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase. *Nat Genet* 10(1):61-66.
- Armstrong RB, Saubert CWt, Seeherman HJ, Taylor CR. 1982. Distribution of fiber types in locomotory muscles of dogs. *Am J Anat* 163(1):87-98.
- Ausborn J, Wolf H, Stein W. 2009. The interaction of positive and negative sensory feedback loops in dynamic regulation of a motor pattern. *Journal of computational neuroscience* 27(2):245-257.
- Averill DR, Jr. 1973. Degenerative myelopathy in the aging German Shepherd dog: clinical and pathologic findings. *J Am Vet Med Assoc* 162(12):1045-1051.
- Awano T, Johnson GS, Wade CM, Katz ML, Johnson GC, Taylor JF, Perloski M, Biagi T, Baranowska I, Long S, March PA, Olby NJ, Shelton GD, Khan S, O'Brien DP, Lindblad-Toh K, Coates JR. 2009. Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 106(8):2794-2799.

- Baloh RH, Rakowicz W, Gardner R, Pestronk A. 2007. Frequent atrophic groups with mixed-type myofibers is distinctive to motor neuron syndromes. *Muscle Nerve* 36(1):107-110.
- Bannister WH, Bannister JV, Barra D, Bond J, Bossa F. 1991. Evolutionary aspects of superoxide dismutase: the copper/zinc enzyme. *Free Radic Res Commun* 12-13 Pt 1:349-361.
- Beal MF, Ferrante RJ, Browne SE, Matthews RT, Kowall NW, Brown RH, Jr. 1997. Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann Neurol* 42(4):644-654.
- Beckman JS, Carson M, Smith CD, Koppenol WH. 1993. ALS, SOD and peroxynitrite. *Nature* 364(6438):584.
- Beers DR, Henkel JS, Xiao Q, Zhao W, Wang J, Yen AA, Siklos L, McKercher SR, Appel SH. 2006. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 103(43):16021-16026.
- Benatar M. 2007. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis* 26(1):1-13.
- Bensimon G, Lacomblez L, Meininger V. 1994. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* 330(9):585-591.
- Boillee S, Vande Velde C, Cleveland DW. 2006a. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron* 52(1):39-59.
- Boillee S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW. 2006b. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312(5778):1389-1392.
- Boissinot M, Karnas S, Lepock JR, Cabelli DE, Tainer JA, Getzoff ED, Hallewell RA. 1997. Function of the Greek key connection analysed using circular permutants of superoxide dismutase. *EMBO J* 16(9):2171-2178.
- Braund KG, McGuire JA, Lincoln CE. 1982a. Age-related changes in peripheral nerves of the dog. II. A morphologic and morphometric study of cross-sectional nerve. *Vet Pathol* 19(4):379-398.
- Braund KG, McGuire JA, Lincoln CE. 1982b. Observations on normal skeletal muscle of mature dogs: a cytochemical, histochemical, and morphometric study. *Vet Pathol* 19(6):577-595.
- Brinkmann JR, Andres P, Mendoza M, Sanjak M. 1997. Guidelines for the use and performance of quantitative outcome measures in ALS clinical trials. *J Neurol Sci* 147(1):97-111.
- Brooks BR. 1994. El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. Subcommittee on Motor Neuron Diseases/Amyotrophic Lateral Sclerosis of the World Federation of Neurology Research Group on Neuromuscular Diseases and the El Escorial "Clinical limits of amyotrophic lateral sclerosis" workshop contributors. *J Neurol Sci* 124 Suppl:96-107.

- Brooks BR, Miller RG, Swash M, Munsat TL. 2000. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 1(5):293-299.
- Brujin LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. 1997. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18(2):327-338.
- Brujin LI, Houseweart MK, Kato S, Anderson KL, Anderson SD, Ohama E, Reaume AG, Scott RW, Cleveland DW. 1998. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* 281(5384):1851-1854.
- Buller AJ, Eccles JC, Eccles RM. 1960. Interactions between motoneurons and muscles in respect of the characteristic speeds of their responses. *The Journal of physiology* 150:417-439.
- Charcot J-M JA. 1869. Deuxcas d'atrophie musculaire progressive avec lesions del substance grise et de faisceaux anterolateraux de moelle epiniere. *Arch Phusiol Norm Pathol*:354-357.
- Clement AM, Nguyen MD, Roberts EA, Garcia ML, Boillee S, Rule M, McMahon AP, Doucette W, Siwek D, Ferrante RJ, Brown RH, Jr., Julien JP, Goldstein LS, Cleveland DW. 2003. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302(5642):113-117.
- Cleveland DW, Rothstein JD. 2001. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat Rev Neurosci* 2(11):806-819.
- Coates JR, March PA, Oglesbee M, Ruaux CG, Olby NJ, Berghaus RD, O'Brien DP, Keating JH, Johnson GS, Williams DA. 2007. Clinical characterization of a familial degenerative myelopathy in Pembroke Welsh Corgi dogs. *J Vet Intern Med* 21(6):1323-1331.
- Coates JR, Wininger FA. 2010. Canine degenerative myelopathy. *Vet Clin North Am Small Anim Pract* 40(5):929-950.
- Crisp MJ, Beckett J, Coates JR, Miller TM. 2013. Canine degenerative myelopathy: Biochemical characterization of superoxide dismutase 1 in the first naturally occurring non-human amyotrophic lateral sclerosis model. *Exp Neurol* 248C:1-9.
- Czaplinski A, Yen AA, Appel SH. 2006. Forced vital capacity (FVC) as an indicator of survival and disease progression in an ALS clinic population. *J Neurol Neurosurg Psychiatry* 77(3):390-392.
- Dadon-Nachum M, Melamed E, Offen D. 2011. The "dying-back" phenomenon of motor neurons in ALS. *J Mol Neurosci* 43(3):470-477.
- Dal Canto MC, Gurney ME. 1994. Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. *Am J Pathol* 145(6):1271-1279.
- Dal Canto MC, Gurney ME. 1995. Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing

- wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). *Brain Res* 676(1):25-40.
- Dal Canto MC, Gurney ME. 1997. A low expressor line of transgenic mice carrying a mutant human Cu,Zn superoxide dismutase (SOD1) gene develops pathological changes that most closely resemble those in human amyotrophic lateral sclerosis. *Acta Neuropathol* 93(6):537-550.
- de Carvalho M, Pinto S, Swash M. 2010. Association of paraspinal and diaphragm denervation in ALS. *Amyotroph Lateral Scler* 11(1-2):63-66.
- DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R. 2011. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 72(2):245-256.
- Deng HX, Chen W, Hong ST, Boycott KM, Gorrie GH, Siddique N, Yang Y, Fecto F, Shi Y, Zhai H, Jiang H, Hirano M, Rampersaud E, Jansen GH, Donkervoort S, Bigio EH, Brooks BR, Ajroud K, Sufit RL, Haines JL, Mugnaini E, Pericak-Vance MA, Siddique T. 2011. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature* 477(7363):211-215.
- Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, Getzoff ED, Hu P, Herzfeldt B, Roos RP, et al. 1993. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* 261(5124):1047-1051.
- Deng HX, Shi Y, Furukawa Y, Zhai H, Fu R, Liu E, Gorrie GH, Khan MS, Hung WY, Bigio EH, Lukas T, Dal Canto MC, O'Halloran TV, Siddique T. 2006. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in mitochondria. *Proc Natl Acad Sci U S A* 103(18):7142-7147.
- Dewil M, dela Cruz VF, Van Den Bosch L, Robberecht W. 2007. Inhibition of p38 mitogen activated protein kinase activation and mutant SOD1(G93A)-induced motor neuron death. *Neurobiol Dis* 26(2):332-341.
- di Trapani G, David P, La Cara A, Servidei S, Tonali P. 1986. Morphological studies of sural nerve biopsies in the pseudopolyneuropathic form of amyotrophic lateral sclerosis. *Clin Neuropathol* 5(3):134-138.
- Dobrowolny G, Aucello M, Rizzuto E, Beccafico S, Mammucari C, Boncompagni S, Belia S, Wannenens F, Nicoletti C, Del Prete Z, Rosenthal N, Molinaro M, Protasi F, Fano G, Sandri M, Musaro A. 2008. Skeletal muscle is a primary target of SOD1G93A-mediated toxicity. *Cell Metab* 8(5):425-436.
- Dyck PJ, Stevens JC, Mulder DW, Espinosa RE. 1975. Frequency of nerve fiber degeneration of peripheral motor and sensory neurons in amyotrophic lateral sclerosis. Morphometry of deep and superficial peroneal nerves. *Neurology* 25(8):781-785.

- Fang F, Kwee LC, Allen KD, Umbach DM, Ye W, Watson M, Keller J, Oddone EZ, Sandler DP, Schmidt S, Kamel F. 2010. Association between blood lead and the risk of amyotrophic lateral sclerosis. *Am J Epidemiol* 171(10):1126-1133.
- Ferrante RJ, Shinobu LA, Schulz JB, Matthews RT, Thomas CE, Kowall NW, Gurney ME, Beal MF. 1997. Increased 3-nitrotyrosine and oxidative damage in mice with a human copper/zinc superoxide dismutase mutation. *Ann Neurol* 42(3):326-334.
- Fischer LR, Brotherton T, Glass JD. 2013. Axonal degeneration in the peripheral nervous system: Implications for the pathogenesis of amyotrophic lateral sclerosis. *Exp Neurol*.
- Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, Khan J, Polak MA, Glass JD. 2004. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol* 185(2):232-240.
- Fischer LR, Li Y, Asress SA, Jones DP, Glass JD. 2012. Absence of SOD1 leads to oxidative stress in peripheral nerve and causes a progressive distal motor axonopathy. *Exp Neurol* 233(1):163-171.
- Flood DG, Reaume AG, Gruner JA, Hoffman EK, Hirsch JD, Lin YG, Dorfman KS, Scott RW. 1999. Hindlimb motor neurons require Cu/Zn superoxide dismutase for maintenance of neuromuscular junctions. *Am J Pathol* 155(2):663-672.
- Forsberg K, Jonsson PA, Andersen PM, Bergemalm D, Graffmo KS, Hultdin M, Jacobsson J, Rosquist R, Marklund SL, Brannstrom T. 2010. Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patients. *PLoS One* 5(7):e11552.
- Frey D, Schneider C, Xu L, Borg J, Spooren W, Caroni P. 2000. Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *J Neurosci* 20(7):2534-2542.
- Fridovich I. 1975. Superoxide dismutases. *Annu Rev Biochem* 44:147-159.
- Fridovich I. 1986. Superoxide dismutases. *Adv Enzymol Relat Areas Mol Biol* 58:61-97.
- Furukawa Y, O'Halloran TV. 2006. Posttranslational modifications in Cu,Zn-superoxide dismutase and mutations associated with amyotrophic lateral sclerosis. *Antioxid Redox Signal* 8(5-6):847-867.
- Garruto RM, Shankar SK, Yanagihara R, Salazar AM, Amyx HL, Gajdusek DC. 1989. Low-calcium, high-aluminum diet-induced motor neuron pathology in cynomolgus monkeys. *Acta Neuropathol* 78(2):210-219.
- Gay S, Jublanc E, Bonnieu A, Bacou F. 2012. Myostatin deficiency is associated with an increase in number of total axons and motor axons innervating mouse tibialis anterior muscle. *Muscle Nerve* 45(5):698-704.
- Giannini F, Battistini S, Mancuso M, Greco G, Ricci C, Volpi N, Del Corona A, Piazza S, Siciliano G. 2010. D90A-SOD1 mutation in ALS: The first report of heterozygous Italian patients and unusual findings. *Amyotroph Lateral Scler* 11(1-2):216-219.
- Gitcho MA, Baloh RH, Chakraborty S, Mayo K, Norton JB, Levitch D, Hatanpaa KJ, White CL, 3rd, Bigio EH, Caselli R, Baker M, Al-Lozi MT, Morris JC, Pestronk A, Rademakers R, Goate AM, Cairns NJ. 2008. TDP-43 A315T mutation in familial motor neuron disease. *Ann Neurol* 63(4):535-538.

- Gong YH, Parsadanian AS, Andreeva A, Snider WD, Elliott JL. 2000. Restricted expression of G86R Cu/Zn superoxide dismutase in astrocytes results in astrogliosis but does not cause motoneuron degeneration. *J Neurosci* 20(2):660-665.
- Gordon PH, Meininger V. 2011. How can we improve clinical trials in amyotrophic lateral sclerosis? *Nat Rev Neurol* 7(11):650-654.
- Gould TW, Buss RR, Vinsant S, Prevette D, Sun W, Knudson CM, Milligan CE, Oppenheim RW. 2006. Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. *J Neurosci* 26(34):8774-8786.
- Green SL, Tolwani RJ, Varma S, Quignon P, Galibert F, Cork LC. 2002. Structure, chromosomal location, and analysis of the canine Cu/Zn superoxide dismutase (SOD1) gene. *J Hered* 93(2):119-124.
- Griffiths IR, Duncan ID. 1975. Chronic degenerative radiculomyelopathy in the dog. *J Small Anim Pract* 16(8):461-471.
- Guegan C, Vila M, Rosoklija G, Hays AP, Przedborski S. 2001. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J Neurosci* 21(17):6569-6576.
- Guo YS, Wu DX, Wu HR, Wu SY, Yang C, Li B, Bu H, Zhang YS, Li CY. 2009. Sensory involvement in the SOD1-G93A mouse model of amyotrophic lateral sclerosis. *Experimental & molecular medicine* 41(3):140-150.
- Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, et al. 1994. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264(5166):1772-1775.
- Hanyu N, Oguchi K, Yanagisawa N, Tsukagoshi H. 1982. Degeneration and regeneration of ventral root motor fibers in amyotrophic lateral sclerosis. Morphometric studies of cervical ventral roots. *Journal of the Neurological Sciences* 55(1):99-115.
- Harding AE, Thomas PK, Baraitser M, Bradbury PG, Morgan-Hughes JA, Ponsford JR. 1982. X-linked recessive bulbospinal neuronopathy: a report of ten cases. *J Neurol Neurosurg Psychiatry* 45(11):1012-1019.
- Heads T, Pollock M, Robertson A, Sutherland WH, Allpress S. 1991. Sensory nerve pathology in amyotrophic lateral sclerosis. *Acta Neuropathol* 82(4):316-320.
- Hegedus J, Putman CT, Gordon T. 2007. Time course of preferential motor unit loss in the SOD1 G93A mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* 28(2):154-164.
- Henriques A, Pitzer C, Schneider A. 2010. Characterization of a novel SOD-1(G93A) transgenic mouse line with very decelerated disease development. *PLoS One* 5(11):e15445.
- Hirano A. 1991. Cytopathology of amyotrophic lateral sclerosis. *Adv Neurol* 56:91-101.
- Hishikawa N, Niwa J, Doyu M, Ito T, Ishigaki S, Hashizume Y, Sobue G. 2003. Dofin localizes to the ubiquitinated inclusions in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and amyotrophic lateral sclerosis. *Am J Pathol* 163(2):609-619.

- Hitsumoto H, Przeborski S, Gordon PH. 2006. Amyotrophic lateral Sclerosis. New York: Taylor and Francis.
- Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. 2002. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A* 99(3):1604-1609.
- Isaacs JD, Dean AF, Shaw CE, Al-Chalabi A, Mills KR, Leigh PN. 2007. Amyotrophic lateral sclerosis with sensory neuropathy: part of a multisystem disorder? *J Neurol Neurosurg Psychiatry* 78(7):750-753.
- Jaarsma D, Haasdijk ED, Grashorn JA, Hawkins R, van Duijn W, Verspaget HW, London J, Holstege JC. 2000. Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes mitochondrial vacuolization, axonal degeneration, and premature motoneuron death and accelerates motoneuron disease in mice expressing a familial amyotrophic lateral sclerosis mutant SOD1. *Neurobiol Dis* 7(6 Pt B):623-643.
- Johnson F, Giulivi C. 2005. Superoxide dismutases and their impact upon human health. *Mol Aspects Med* 26(4-5):340-352.
- Johnson MA, Sideri G, Weightman D, Appleton D. 1973. A comparison of fibre size, fibre type constitution and spatial fibre type distribution in normal human muscle and in muscle from cases of spinal muscular atrophy and from other neuromuscular disorders. *J Neurol Sci* 20(4):345-361.
- Jonsson PA, Graffmo KS, Brannstrom T, Nilsson P, Andersen PM, Marklund SL. 2006. Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. *J Neuropathol Exp Neurol* 65(12):1126-1136.
- Kato S, Horiuchi S, Liu J, Cleveland DW, Shibata N, Nakashima K, Nagai R, Hirano A, Takikawa M, Kato M, Nakano I, Ohama E. 2000. Advanced glycation endproduct-modified superoxide dismutase-1 (SOD1)-positive inclusions are common to familial amyotrophic lateral sclerosis patients with SOD1 gene mutations and transgenic mice expressing human SOD1 with a G85R mutation. *Acta Neuropathol* 100(5):490-505.
- Kawamura Y, Dyck PJ, Shimono M. 1981. Morphometric comparison of the vulnerability of peripheral motor and sensory neurons in amyotrophic lateral sclerosis. *Journal of Neuropathology and Experimental Neurology* 40(6):667-675.
- Khoris J, Moulard B, Briolotti V, Hayer M, Durieux A, Clavelou P, Malafosse A, Rouleau GA, Camu W. 2000. Coexistence of dominant and recessive familial amyotrophic lateral sclerosis with the D90A Cu,Zn superoxide dismutase mutation within the same country. *Eur J Neurol* 7(2):207-211.
- Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC. 2011. Amyotrophic lateral sclerosis. *Lancet* 377(9769):942-955.
- Laaksovirta H, Peuralinna T, Schymick JC, Scholz SW, Lai SL, Myllykangas L, Sulkava R, Jansson L, Hernandez DG, Gibbs JR, Nalls MA, Heckerman D, Tienari PJ, Traynor

- BJ. 2010. Chromosome 9p21 in amyotrophic lateral sclerosis in Finland: a genome-wide association study. *Lancet Neurol* 9(10):978-985.
- Liddell EGT, Sherrington C. 1924. Reflexes in Response to Stretch (Myotatic Reflexes). *Proceedings of the Royal Society of London Series B, Containing Papers of a Biological Character* 96(675):212-242.
- Lino MM, Schneider C, Caroni P. 2002. Accumulation of SOD1 mutants in postnatal motoneurons does not cause motoneuron pathology or motoneuron disease. *J Neurosci* 22(12):4825-4832.
- Lobsiger CS, Garcia ML, Ward CM, Cleveland DW. 2005. Altered axonal architecture by removal of the heavily phosphorylated neurofilament tail domains strongly slows superoxide dismutase 1 mutant-mediated ALS. *Proc Natl Acad Sci U S A* 102(29):10351-10356.
- March PA, Coates JR, Abyad RJ, Williams DA, O'Brien DP, Olby NJ, Keating JH, Oglesbee M. 2009. Degenerative myelopathy in 18 Pembroke Welsh Corgi dogs. *Vet Pathol* 46(2):241-250.
- Marcuzzo S, Zucca I, Mastropietro A, de Rosbo NK, Cavalcante P, Tartari S, Bonanno S, Preite L, Mantegazza R, Bernasconi P. 2011. Hind limb muscle atrophy precedes cerebral neuronal degeneration in G93A-SOD1 mouse model of amyotrophic lateral sclerosis: a longitudinal MRI study. *Exp Neurol* 231(1):30-37.
- Mariotti C, Castellotti B, Pareyson D, Testa D, Eoli M, Antozzi C, Silani V, Marconi R, Tezzon F, Siciliano G, Marchini C, Gellera C, Donato SD. 2000. Phenotypic manifestations associated with CAG-repeat expansion in the androgen receptor gene in male patients and heterozygous females: a clinical and molecular study of 30 families. *Neuromuscul Disord* 10(6):391-397.
- Martin LJ. 1999. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. *J Neuropathol Exp Neurol* 58(5):459-471.
- Matsumoto G, Stojanovic A, Holmberg CI, Kim S, Morimoto RI. 2005. Structural properties and neuronal toxicity of amyotrophic lateral sclerosis-associated Cu/Zn superoxide dismutase 1 aggregates. *J Cell Biol* 171(1):75-85.
- McCord JM, Fridovich I. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 244(22):6049-6055.
- Mentis GZ, Blivis D, Liu W, Drobac E, Crowder ME, Kong L, Alvarez FJ, Sumner CJ, O'Donovan MJ. 2011. Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. *Neuron* 69(3):453-467.
- Miller CJ. 2007. Approach to the respiratory patient. *Vet Clin North Am Small Anim Pract* 37(5):861-878, v.
- Miller TM, Pestronk A, David W, Rothstein J, Simpson E, Appel SH, Andres PL, Mahoney K, Allred P, Alexander K, Ostrow LW, Schoenfeld D, Macklin EA, Norris DA, Manousakis G, Crisp M, Smith R, Bennett CF, Bishop KM, Cudkovic ME. 2013. An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *Lancet Neurol* 12(5):435-442.

- Mizoule J, Meldrum B, Mazadier M, Croucher M, Ollat C, Uzan A, Legrand JJ, Gueremy C, Le Fur G. 1985. 2-Amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission--I. Anticonvulsant properties. *Neuropharmacology* 24(8):767-773.
- Morgan BR, Coates JR, Johnson JC, Bujnak AC, Katz ML. 2013. Characterization of Intercostal Muscle Pathology in Canine Degenerative Myelopathy: A Disease Model for Amyotrophic Lateral Sclerosis. *J Neuroscience Res* In press.
- Morren JA, Galvez-Jimenez N. 2012. Current and prospective disease-modifying therapies for amyotrophic lateral sclerosis. *Expert Opin Investig Drugs* 21(3):297-320.
- Mulder DW, Bushek W, Spring E, Karnes J, Dyck PJ. 1983. Motor neuron disease (ALS): evaluation of detection thresholds of cutaneous sensation. *Neurology* 33(12):1625-1627.
- Mulder DW, Kurland LT, Offord KP, Beard CM. 1986. Familial adult motor neuron disease: amyotrophic lateral sclerosis. *Neurology* 36(4):511-517.
- Muller FL, Song W, Liu Y, Chaudhuri A, Pieke-Dahl S, Strong R, Huang TT, Epstein CJ, Roberts LJ, 2nd, Csete M, Faulkner JA, Van Remmen H. 2006. Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free radical biology & medicine* 40(11):1993-2004.
- Munch C, Bertolotti A. 2010. Exposure of hydrophobic surfaces initiates aggregation of diverse ALS-causing superoxide dismutase-1 mutants. *J Mol Biol* 399(3):512-525.
- Ogawa M, Uchida K, Park ES, Kamishina H, Sasaki J, Chang HS, Yamato O, Nakayama H. 2011. Immunohistochemical observation of canine degenerative myelopathy in two Pembroke Welsh Corgi dogs. *J Vet Med Sci* 73(10):1275-1279.
- Ogawa M, Uchida K, Yamato O, Inaba M, Uddin MM, Nakayama H. 2013. Neuronal Loss and Decreased GLT-1 Expression Observed in the Spinal Cord of Pembroke Welsh Corgi Dogs With Canine Degenerative Myelopathy. *Vet Pathol*.
- Onesto E, Rusmini P, Crippa V, Ferri N, Zito A, Galbiati M, Poletti A. 2011. Muscle cells and motoneurons differentially remove mutant SOD1 causing familial amyotrophic lateral sclerosis. *J Neurochem* 118(2):266-280.
- Perrie WT, Lee GT, Curtis EM, Sparke J, Buller JR, Rossi ML. 1993. Changes in the myelinated axons of femoral nerve in amyotrophic lateral sclerosis. *Journal of Neural Transmission, Supplement*(39):223-233.
- Pramatarova A, Laganier J, Roussel J, Brisebois K, Rouleau GA. 2001. Neuron-specific expression of mutant superoxide dismutase 1 in transgenic mice does not lead to motor impairment. *J Neurosci* 21(10):3369-3374.
- Prudencio M, Durazo A, Whitelegge JP, Borchelt DR. 2009. Modulation of mutant superoxide dismutase 1 aggregation by co-expression of wild-type enzyme. *J Neurochem* 108(4):1009-1018.
- Prudencio M, Durazo A, Whitelegge JP, Borchelt DR. 2010. An examination of wild-type SOD1 in modulating the toxicity and aggregation of ALS-associated mutant SOD1. *Hum Mol Genet* 19(24):4774-4789.

- Pugdahl K, Fuglsang-Frederiksen A, de Carvalho M, Johnsen B, Fawcett PR, Labarre-Vila A, Liguori R, Nix WA, Schofield IS. 2007. Generalised sensory system abnormalities in amyotrophic lateral sclerosis: a European multicentre study. *J Neurol Neurosurg Psychiatry* 78(7):746-749.
- Rakhit R, Chakrabartty A. 2006. Structure, folding, and misfolding of Cu,Zn superoxide dismutase in amyotrophic lateral sclerosis. *Biochim Biophys Acta* 1762(11-12):1025-1037.
- Ravits J, Laurie P, Fan Y, Moore DH. 2007. Implications of ALS focality: rostral-caudal distribution of lower motor neuron loss postmortem. *Neurology* 68(19):1576-1582.
- Ravits JM, La Spada AR. 2009. ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration. *Neurology* 73(10):805-811.
- Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH, Jr., Scott RW, Snider WD. 1996. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet* 13(1):43-47.
- Ripps ME, Huntley GW, Hof PR, Morrison JH, Gordon JW. 1995. Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 92(3):689-693.
- Robberecht W, Aguirre T, Van den Bosch L, Tilkin P, Cassiman JJ, Matthijs G. 1996. D90A heterozygosity in the SOD1 gene is associated with familial and apparently sporadic amyotrophic lateral sclerosis. *Neurology* 47(5):1336-1339.
- Roelofs-Iverson RA, Mulder DW, Elveback LR, Kurland LT, Molgaard CA. 1984. ALS and heavy metals: a pilot case-control study. *Neurology* 34(3):393-395.
- Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al. 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362(6415):59-62.
- Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16(3):675-686.
- Rothstein JD, Tsai G, Kuncl RW, Clawson L, Cornblath DR, Drachman DB, Pestronk A, Stauch BL, Coyle JT. 1990. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Ann Neurol* 28(1):18-25.
- Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. 1995. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol* 38(1):73-84.
- Rouaux C, Panteleeva I, Rene F, Gonzalez de Aguilar JL, Echaniz-Laguna A, Dupuis L, Menger Y, Boutillier AL, Loeffler JP. 2007. Sodium valproate exerts neuroprotective effects in vivo through CREB-binding protein-dependent mechanisms but does not improve survival in an amyotrophic lateral sclerosis mouse model. *J Neurosci* 27(21):5535-5545.

- Rozanski EA, Hoffman AM. 1999. Pulmonary function testing in small animals. *Clin Tech Small Anim Pract* 14(4):237-241.
- Sasaki S, Maruyama S. 1994. Synapse loss in anterior horn neurons in amyotrophic lateral sclerosis. *Acta Neuropathol* 88(3):222-227.
- Schaefer AM, Sanes JR, Lichtman JW. 2005. A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. *J Comp Neurol* 490(3):209-219.
- Scott S, Kranz JE, Cole J, Lincecum JM, Thompson K, Kelly N, Bostrom A, Theodoss J, Al-Nakhala BM, Vieira FG, Ramasubbu J, Heywood JA. 2008. Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler* 9(1):4-15.
- Sewry CA. 2010. Muscular dystrophies: an update on pathology and diagnosis. *Acta Neuropathol* 120(3):343-358.
- Shaw PJ, Forrest V, Ince PG, Richardson JP, Wastell HJ. 1995. CSF and plasma amino acid levels in motor neuron disease: elevation of CSF glutamate in a subset of patients. *Neurodegeneration* 4(2):209-216.
- Shelton GD, Johnson GC, O'Brien DP, Katz ML, Pesayco JP, Chang BJ, Mizisin AP, Coates JR. 2012. Degenerative myelopathy associated with a missense mutation in the superoxide dismutase 1 (SOD1) gene progresses to peripheral neuropathy in Pembroke Welsh Corgis and Boxers. *J Neurol Sci* 318(1-2):55-64.
- Shen H, Barry DM, Dale JM, Garcia VB, Calcutt NA, Garcia ML. 2011. Muscle pathology without severe nerve pathology in a new mouse model of Charcot-Marie-Tooth disease type 2E. *Hum Mol Genet* 20(13):2535-2548.
- Shibata N, Hirano A, Kobayashi M, Siddique T, Deng HX, Hung WY, Kato T, Asayama K. 1996. Intense superoxide dismutase-1 immunoreactivity in intracytoplasmic hyaline inclusions of familial amyotrophic lateral sclerosis with posterior column involvement. *J Neuropathol Exp Neurol* 55(4):481-490.
- Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, Hung G, Lobsiger CS, Ward CM, McAlonis-Downes M, Wei H, Wancewicz EV, Bennett CF, Cleveland DW. 2006. Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest* 116(8):2290-2296.
- Sobue G, Hashizume Y, Mitsuma T, Takahashi A. 1987. Size-dependent myelinated fiber loss in the corticospinal tract in Shy-Drager syndrome and amyotrophic lateral sclerosis. *Neurology* 37(3):529-532.
- Sobue G, Hashizume Y, Mukai E, Hirayama M, Mitsuma T, Takahashi A. 1989. X-linked recessive bulbospinal neuronopathy. A clinicopathological study. *Brain* 112 (Pt 1):209-232.
- Sobue G, Matsuoka Y, Mukai E, Takayanagi T, Sobue I, Hashizume Y. 1981. Spinal and cranial motor nerve roots in amyotrophic lateral sclerosis and X-linked recessive bulbospinal muscular atrophy: morphometric and teased-fiber study. *Acta Neuropathol* 55(3):227-235.
- Soraru G, D'Ascenzo C, Nicolao P, Volpe M, Martignago S, Palmieri A, Romeo V, Koutsikos K, Piccione F, Cima V, Pegoraro E, Angelini C. 2008a. Muscle

- histopathology in upper motor neuron-dominant amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis* 9(5):287-293.
- Soraru G, D'Ascenzo C, Polo A, Palmieri A, Baggio L, Vergani L, Gellera C, Moretto G, Pegoraro E, Angelini C. 2008b. Spinal and bulbar muscular atrophy: skeletal muscle pathology in male patients and heterozygous females. *J Neurol Sci* 264(1-2):100-105.
- Spencer PS, Nunn PB, Hugon J, Ludolph A, Roy DN. 1986. Motorneuron disease on Guam: possible role of a food neurotoxin. *Lancet* 1(8487):965.
- Sunico CR, Dominguez G, Garcia-Verdugo JM, Osta R, Montero F, Moreno-Lopez B. 2011. Reduction in the motoneuron inhibitory/excitatory synaptic ratio in an early-symptomatic mouse model of amyotrophic lateral sclerosis. *Brain Pathol* 21(1):1-15.
- Swash M. 2007. We have a problem: why have ALS trials been negative? *Amyotroph Lateral Scler* 8(5):259.
- Swynghedauw B. 1986. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiological reviews* 66(3):710-771.
- Tainer JA, Getzoff ED, Richardson JS, Richardson DC. 1983. Structure and mechanism of copper, zinc superoxide dismutase. *Nature* 306(5940):284-287.
- Telerman-Toppet N, Coers C. 1978. Motor innervation and fiber type pattern in amyotrophic lateral sclerosis and in Charcot-Marie-Tooth disease. *Muscle and Nerve* 1(2):133-139.
- Turner BJ, Talbot K. 2008. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog Neurobiol* 85(1):94-134.
- Victor Dubowitz CAS. 2007. *Muscle Biopsy: A Practical Approach*: Saunders Elsevier.
- Wang J, Slunt H, Gonzales V, Fromholt D, Coonfield M, Copeland NG, Jenkins NA, Borchelt DR. 2003. Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature. *Hum Mol Genet* 12(21):2753-2764.
- Watanabe M, Dykes-Hoberg M, Culotta VC, Price DL, Wong PC, Rothstein JD. 2001. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol Dis* 8(6):933-941.
- Wetts R, Vaughn JE. 1996. Differential vulnerability of two subsets of spinal motor neurons in amyotrophic lateral sclerosis. *Exp Neurol* 141(2):248-255.
- Wininger FA, Zeng R, Johnson GS, Katz ML, Johnson GC, Bush WW, Jarboe JM, Coates JR. 2011. Degenerative Myelopathy in a Bernese Mountain Dog with a Novel SOD1 Missense Mutation. *J Vet Intern Med* 25(5):1166-1170.
- Winter SM, Claus A, Oberwittler C, Volkel H, Wenzler S, Ludolph AC. 2000. Recessively inherited amyotrophic lateral sclerosis: a Germany family with the D90A CuZn-SOD mutation. *J Neurol* 247(10):783-786.
- Wong M, Martin LJ. 2010. Skeletal muscle-restricted expression of human SOD1 causes motor neuron degeneration in transgenic mice. *Hum Mol Genet* 19(11):2284-2302.

- Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. 1995. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14(6):1105-1116.
- Zetterstrom P, Stewart HG, Bergemalm D, Jonsson PA, Graffmo KS, Andersen PM, Brannstrom T, Oliveberg M, Marklund SL. 2007. Soluble misfolded subfractions of mutant superoxide dismutase-1s are enriched in spinal cords throughout life in murine ALS models. *Proc Natl Acad Sci U S A* 104(35):14157-14162.
- Zhou J, Yi J, Fu R, Liu E, Siddique T, Rios E, Deng HX. 2010. Hyperactive intracellular calcium signaling associated with localized mitochondrial defects in skeletal muscle of an animal model of amyotrophic lateral sclerosis. *J Biol Chem* 285(1):705-712.

VITA

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