DEVELOPMENT OF A MOTOR UNIT NUMBER ESTIMATION TECHNIQUE

IN NORMAL DOGS:

A POTENTIAL BIOMARKER

FOR CANINE DEGENERATIVE MYELOPATHY

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A Thesis

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the Faculty of the Graduate School

at the University of Missouri-Columbia

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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DEVELOPMENT OF A MOTOR UNIT NUMBER ESTIMATION TECHNIQUE IN NORMAL DOGS: A POTENTIAL BIOMARKER FOR CANINE DEGENERATIVE MYELOPATHY

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<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<tr>
<td>CMAP</td>
<td>compound muscle action potential</td>
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<td>DM</td>
<td>degenerative myelopathy</td>
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<tr>
<td>EMG</td>
<td>electromyography</td>
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<tr>
<td>EDB</td>
<td>extensor digitorum brevis</td>
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<tr>
<td>LMN</td>
<td>lower motor neuron</td>
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<tr>
<td>MNCV</td>
<td>motor nerve conduction velocity</td>
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<tr>
<td>MUNE</td>
<td>motor unit number estimation</td>
</tr>
<tr>
<td>MUP</td>
<td>motor unit potential</td>
</tr>
<tr>
<td>UMN</td>
<td>upper motor neuron</td>
</tr>
<tr>
<td>SMUP</td>
<td>single motor unit potential</td>
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DEVELOPMENT OF A MOTOR UNIT NUMBER ESTIMATION TECHNIQUE 
IN NORMAL DOGS:
A POTENTIAL BIOMARKER FOR CANINE DEGENERATIVE MYELOPATHY

ABSTRACT

Motor unit number estimation (MUNE) is an electrophysiologic technique for quantifying the lower motor neuron (LMN) system. MUNE has proven useful in evaluating and monitoring neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS). Recently a missense mutation in the canine superoxide dismutase 1 (SOD1) gene has been shown to be a risk factor for canine degenerative myelopathy (DM) suggesting homology to familial SOD1 ALS. To date, the LMN component of DM has not been well characterized or quantified. The modified incremental stimulation MUNE technique was applied to the sciatic-deep peroneal nerve branch with bilateral recordings at the extensor digitorum brevis muscle of 17 clinically normal dogs. Mean (± SD) value for the entire MUNE pool was 51 ± 21 with a range from 8 to 154. No statistically significant difference was noted between pelvic limbs (P=0.14) or between different age groups (< 7 years old or ≥ 7 years old) (P=0.17). Test-retest reliability was assessed for trials performed under different anesthetic episodes (intermittent) versus trials performed under the same anesthetic episode (consecutive). The intraclass correlation coefficients for consecutive and intermittent MUNE evaluations were 0.73 and 0.65, respectively. These results provide preliminary reference ranges for normal dogs and document the potential utility of EDB modified incremental stimulation MUNE for longitudinal monitoring of lower motor neuron loss in DM affected dogs.
CHAPTER 1

INTRODUCTION

First described by Averill in 1973, canine degenerative myelopathy (DM) is an adult-onset, progressive, non-painful neurodegenerative disorder affecting many pure-bred as well as mixed-breed dogs.\(^1\) It has historically been considered an upper motor neuron (UMN) disease but recent reports on DM-affected Pembroke Welsh Corgis that were supportively cared for beyond the UMN paraparesis/plegia described the manifestation of LMN signs and brainstem signs as well as thoracic limb involvement.\(^2-3\)

In dogs with advanced DM, nerve specimens have demonstrated axonal degeneration while corresponding muscle specimens have changes typical of denervation atrophy.\(^4\) In 2009, Awano and colleagues described the use of genome-wide association mapping to identify an E40K missense mutation in the superoxide dismutase 1 (\(SOD1\)) gene on canine chromosome 31 of DM-affected dogs.\(^2\) This mutation is now considered a risk factor for the development of DM. Immunohistochemistry of DM-affected spinal cords when compared to age-matched controls had ventral gray horn cell bodies positively stained for anti-\(SOD1\) antibodies. \(SOD1\)-positive cytoplasmic inclusions are the hallmark of \(SOD1\)-associated amyotrophic lateral sclerosis (ALS) in humans.\(^5\) DM appears to be the first reported spontaneously occurring animal model of some forms of ALS.

Animal models are critical for investigating mechanisms of disease as well as evaluating novel therapeutic strategies. Although much information regarding ALS has been the result of the work performed on transgenic mouse models, there are limitations to their translational ability. Dogs can serve as an ideal intermediate-sized model
between rodents and humans. In addition, a spontaneous canine model offers a ready clinical population for evaluation of therapeutic interventions in a setting that closely mimics human clinical trials. This has proven a successful strategy in the evaluation and development of chemotherapeutic agents targeting various types of cancers homologous between dogs and humans.6

Various UMN and LMN surrogate markers are used as outcome measures in humans for longitudinal study of therapeutic efficacies and disease progression.7-9 Quantifiable biomarkers for DM have yet to be established. In addition, the LMN signs associated with DM have yet to be well described. To establish DM as an animal model of ALS and to establish a biomarker for study of efficacy of therapeutics, we need an objective and quantifiable measure of LMN involvement. The goal of this study was to establish a practical, reproducible, and quantifiable technique to evaluate the motor unit in normal dogs and, thus, serve as a potential biomarker for study of DM-affected dogs.
CHAPTER 2

COMPARITIVE MEDICINE: DEGENERATIVE MYELOPATHY AND
AMYOTROPHIC LATERAL SCLEROSIS

I. Degenerative Myelopathy

Canine degenerative myelopathy was first described in 1973 in twenty-two dogs, twenty of which were German Shepherd Dogs (GSD).\(^1\) It was described as a progressive non-painful adult-onset proprioceptive ataxia of the pelvic limbs with concurrent UMN paraparesis. No underlying cause such as intervertebral disc disease or neoplasia was observed. The author concluded that these dogs exhibited a primary spinal cord degeneration of unknown etiology.\(^1\) The author speculated as to the possibility of a vitamin deficiency or familial axonal degeneration, two conditions observed in man.

In 1975, the term *chronic degenerative radiculomyelopathy* was used to describe the same condition based on histopathologic evaluation in a set number of dogs.\(^10\) Signalment, clinical signs and progression to euthanasia were fairly similar to those described previously. Electromyography and nerve conduction studies were performed on eight of the dogs and revealed no abnormalities.\(^10\) The implication of unremarkable electrodiagnostics appeared to rule out both a myopathy and a demyelinating neuropathy.

i. Etiologic Investigations

Braund and Vandevelde assessed whether the GSD myelopathy was a dying-back process.\(^11\) Dying back neuropathy is the common term for a distal axonopathy. It is often secondary to a metabolic or toxic insult that interrupts axonal transport.\(^12-13\)
Anterograde transport disruption interferes with delivery of substrates necessary for neurotransmitter synthesis in the nerve terminal while retrograde transport disruption leads to accumulation of metabolic waste products in the nerve terminal. Dying back lesions are typically symmetrical and ascend towards the neuronal cell body. Lesions in the DM-affected dogs occurred predominantly in the white matter tracts along the thoracic and lumbosacral spinal cord segments. The lesions did not appear continuous or symmetrical, as typically observed with distal axonopathies. The authors hypothesized that a genetic cause might be a predisposing factor in the GSDs.

In 1980, a different research group considered the role of the immune system in DM. They hypothesized that the demyelination observed might be similar to other demyelinating conditions such as multiple sclerosis or experimental allergic encephalomyelitis. Waxman et al. evaluated peripheral lymphocyte response to mitogens in vitro from both affected and non-affected dogs. The data indicated a blunted response in the affected dogs to both concanavalin A and phytohemagglutinin. Interestingly, lymph node and splenic lymphocyte populations did not have a decreased response when subjected to the same T-dependent mitogens. Peripherally-derived lymphocytes were incubated with lymph node and splenic tissue and had the same mitogens applied. Results indicated that there was an agent in the peripheral blood that was causing response suppression. The authors’ postulated that an unknown aberrant suppressor cell population was allowing an infectious agent to propagate in the central nervous system leading to progressive myelopathy. Although this suppression was illustrated in vitro, it did not correlate with the normal response seen in the spleen and lymph nodes of affected dogs. Waxman’s colleague, Clemmons further described elevations in circulating
immune complexes in affected dogs as compared to normal dogs (unpublished data).\textsuperscript{16-17} Unfortunately, the results from these studies were never published and thus their claims could not be corroborated. Another hindrance of this theory was lack of secondary bacterial infections commonly seen in animals that are immunocompromised.

The hypothesis of an immune-mediated cause was further investigated when Barclay and Haines immunohistochemically assessed the spinal cords of DM-affected dogs for immunoglobulin G (IgG) and complement 3 (C3).\textsuperscript{18} Paraffin-embedded tissue samples from three affected dogs and one normal control dog were examined. In DM-affected dogs, increased IgG and C3 staining were associated with areas of increased vascularization proximal to affected areas. These extra-vascular deposits appeared to correspond with areas of myelin loss. There also appeared to be focal, localized deposits of IgG and C3 in areas without apparent lesions or vascularization. The control dog tissue also had stain uptake associated with blood vessels.\textsuperscript{18} No specific DM antigens were detected and the study had limited controls, so no conclusion regarding endogenous versus exogenous etiology could be supported.

\textbf{ii. Species Comparisons}

Similarities between DM and other spinal neurodegenerative disorders in other species, including cattle, horses, and humans, have also been studied throughout the last thirty years.

Bovine progressive degenerative myeloencephalopathy, Weaver syndrome, was first described in 1973 in Brown Swiss cattle.\textsuperscript{19} It occurs throughout North America and Europe. Calves are normal until approximately 6 months of age when paraparesis and
general proprioceptive ataxia in the hindlimbs develop. Thoracic limbs remain normal, but the calves become progressively weaker in the pelvic limbs and are usually euthanized by 22 months of age when they become recumbent. Degeneration of the white matter with the presence of spheroids occurs diffusely along the spinal cord, in brain stem nuclei, and in the cerebellar granular layer. The spheroids suggest an axonal transport dysfunction. Muscle wasting of the hindlimbs may also occur, but no degeneration of the motor unit has been reported. The disease appears to be hereditary with 54% of the cases between 1957 and 1983 linked to five bull sires. An added risk factor may be milk production. No treatment has been found to be effective. Careful breeding protocols have decreased the incidence in the United States. DM differs from bovine progressive myelopathy in age of onset as well as distribution and pathology of the lesions.

Equine degenerative myeloencephalopathy affects various breeds worldwide with a concentration in the northeastern United States. The disease in horses was first described in 1976. Onset is predominantly within the first 12 months of life. Affected horses initially display an UMN tetraparesis that is more pronounced in the pelvic limbs. Disease progression and chronicity can lead to LMN signs with most horses being euthanized by two to three years of age. A genetic predisposition along with concurrent vitamin E deficiency is considered a risk factor for development of clinical signs. Supplementation with vitamin E has been shown to halt disease progression. Vitamin E is a free radical scavenger in plasma membranes. Vitamin E deficiency is thought to potentiate peroxidative processes and increase membrane fragility culminating in cell death.
A second equine disease also linked to vitamin E deficiency is equine motor neuron disease (EMND). The disease differs from equine degenerative myeloencephalopathy in that it exclusively targets the LMN rather than the UMN system. The first cases occurred in 1982 in the northeastern United States, although it has now been reported worldwide.\(^{21,26}\) Disease occurs in older horses and peaks at 16 years of age. The disease occurs with increased prevalence in Quarter horses and Thoroughbreds. The majority of cases occur in horses that are stabled for long periods with no access to green forage. Clinical signs include dramatic weight loss, fasciculations, generalized LMN paresis including low head carriage and a short-strided gait in all four limbs. General proprioceptive ataxia is not a clinical feature. Histologically, there is diffuse loss of motor neuron cell bodies in the brainstem and spinal cord.\(^{26}\) Other histopathologic findings include loss of cell body Nissl substance, neurofilament accumulation at the axon hillock, and cell body swelling. Antemortem test results that correlate with EMND diagnosis include decreased plasma vitamin E levels, abnormalities on electromyography (EMG) of the sacrocaudalis dorsalis medialis muscle, and abnormal nerve biopsy results. EMG abnormalities suggest denervation muscle atrophy and include prolonged insertional activity and presence of fibrillation potentials, and positive-sharp waves. Nerve and muscle biopsies typically show axonal loss with neurogenic atrophy of the type-1 myofibers.\(^{26-27}\) EMND is clinically and histopathologically similar to the human motor neuron disease, progressive muscular atrophy (PMA), a rare sporadic form of ALS. Investigations into the equine SOD1 gene have not revealed mutations homologous to those seen in PMA.\(^{28}\) Similar to equine degenerative myeloencephalopathy,
supplementation with vitamin E will halt disease progression, but the animal is likely to exhibit residual neurologic deficits.

In man, vitamin E levels are often 10% of normal before clinical signs of ataxia develop. Likewise, levels in horses affected by equine degenerative myeloencephalopathy or EMND average approximately 65% of normal.

A study evaluating vitamin E levels in serum of DM-affected dogs showed slightly lower levels when compared to normal dogs. A second study evaluated serum vitamin E concentrations in three different groups: DM-affected and non-affected GSDs as well as unaffected dogs of other breeds. Interestingly, affected dogs had significantly higher levels of α-tocopherol (vitamin E) as compared to unaffected dogs of other breeds but not significantly higher levels than unaffected GSDs. A confounding factor, however, was that many of the affected dogs were concurrently receiving vitamin E supplementation. Given that affected GSDs in Johnston’s study had elevated rather than decreased levels of α-tocopherol in conjunction with progressive signs made it less likely that vitamin E deficiency was an underlying cause.

Vitamin B₁₂ (cobalamin) deficiency has also been shown to affect the central nervous system. B₁₂ is important in essential metabolic pathways, DNA synthesis, and energy production. Deficiency primarily leads to demyelination and gliosis. Humans and monkeys with B₁₂ deficiencies develop clinical signs of T3-L3 myelopathy with corresponding lesions in the mid-thoracic spinal cord. GSDs are commonly affected with malabsorptive syndromes such as exocrine pancreatic insufficiency that may predispose them to develop cobalamin deficiencies. Several studies evaluated B₁₂ levels
in DM-affected GSDs. The dogs were found to be asymptomatic for gastro-intestinal disturbances. Small intestinal biopsies did not reveal changes indicative of pathology. Serum quantitative enzymology, however, did reveal brush border enzyme alterations such as decreased leucyl-2-naphthylamidase and increased γ-glutamyl transferase. B12 levels were only decreased in three of the six dogs. The author speculated as to whether the deficiency was the causative agent of neurological decline or vice versa. Anecdotal reports suggest that supplementation with parenteral cobalamin did not alter progression of clinical signs.

There are numerous reports describing histopathologically confirmed DM in certain breeds such as the GSD, Rhodesian ridgeback, Boxer dog, miniature Poodle, and Chesapeake Bay retriever. Because DM is a spontaneous disease with uniformity in onset of clinical signs and disease progression, an underlying genetic cause has long been suspected. With the advent of the canine genome project as well as advances in genetic methodologies over the last 20 years, the over-represented DM-affected dog breeds were more thoroughly investigated.

In 2007, pedigree analysis was performed on Pembroke Welsh Corgis, including 21 histopathologically-confirmed DM-affected dogs. The pedigree analysis suggested an autosomal recessive pattern of inheritance. DNA samples from DM-affected and non-affected Pembroke Welsh Corgis were also collected and stored. Genome-wide association mapping was employed on samples from 38 histologically-confirmed DM-affected dogs and 17 control dogs. Mapping produced the strongest associations with markers on chromosome CFA31 in a region containing the candidate gene SOD1. Re-sequencing of SOD1 in DNA from normal and DM-affected dogs revealed a missense
mutation in exon 2. Homozygosity for the SOD1:c.118G>A allele is considered a risk factor for the development of DM.  

II. **Amyotrophic Lateral Sclerosis**

ALS is a heterogeneous group of adult onset human diseases characterized by progressive degeneration of the motor neuron system causing advancing weakness and muscle atrophy that culminates in paralysis and death. The disease was first described by the French neurologist Jean-Martin Charcot in 1869. In North America, it is commonly called *Lou Gehrig’s disease*, after the professional baseball player who was diagnosed with the condition in 1939. *Motor neuron disease* has also been advocated as an all-encompassing term for the various clinical manifestations of ALS and diseases thought to be related to ALS.

ALS is the most common motor neuron disease in the world with an overall incidence of approximately 6 in 100,000 people. In certain geographic locales, the prevalence of ALS is significantly higher as in Guam and Kii peninsula, Japan, where 100 to 200 persons per 100,000 are affected.

A majority of ALS cases are sporadic (SALS) with less than 10% being familial (FALS). Advances in genomics in the 1990s led to the discovery of several mutations in a subset of FALS patients. DNA samples from 23 families with 60 ALS-affected members were evaluated using multi-point linkage analysis that revealed a candidate region on the long arm of chromosome 21q22.11 where the *SOD1* gene is located. Polymerase chain reaction and single-stranded conformational polymorphism were applied to affected individuals as well as controls to reveal sequence changes in exons 1
and 2 of the SOD1 gene in 17 of 49 families evaluated. Fourteen different single point mutations were identified with an alanine to valine mutation at exon 1 being the most common. Today, over 150 SOD1 mutations have been identified encompassing approximately 50% of the 153 amino acids that make up the enzyme. There are 108 missense mutations with an exchange of one amino acid while retaining the total of 153 amino acids.

III. E40K and D90A

The clinical description for the E40K canine-associated SOD1 mutation is similar to the human D90A SOD1 mutation. A majority of D90A cases are associated with an autosomal recessive pattern of inheritance, meaning an individual has to be homozygous for the mutant allele for phenotype manifestation. However, ALS has also been identified in D90A heterozygotes. The majority of histologically confirmed DM-affected dogs have been homozygous for the E40K mutation (JR Coates, unpublished observation). The mutation is only considered a risk factor because there are many asymptomatic dogs homozygous for the mutation. More recently, heterozygous carriers have been histologically-confirmed with DM (Coates, unpublished data).

It appears that the D90A and E40K mutations have multifactorial incomplete penetrance. The majority of ALS SOD1 mutations are autosomal dominant. It is possible that unidentified gene modifiers or environmental influences may be rendering protection or exacerbating phenotype manifestation. DM-affected dogs could be used to further investigate the disparity between alleles and phenotypes for both E40K and D90A, especially in regards to mapping modifier loci.
IV. Comparison of Clinical Presentations

Clinical signs for DM have similarities to the El Escorial criteria developed to standardize diagnosis of ALS for research purposes. The characteristic clinical signs of ALS include presence of UMN and LMN system degenerations and the progressive spread of these signs within a region or to other regions, a pathologic continuum of a multisystem disorder. At disease onset, an ALS patient usually presents with only UMN or LMN signs that involve either upper or lower limb muscles or muscles innervated by brain stem (bulbar) nuclei. Thus, these forms of ALS are termed UMN onset, LMN onset, or bulbar onset. One study found a 9% incident of ALS with lower motor neuron signs alone termed progressive muscular atrophy. The most common form, previously known as Charcot’s disease, is spinal-onset ALS with a mix of both UMN and LMN signs that accounts for approximately 75% of reported cases. Other forms include ALS with multi-system involvement (ALS-Dementia) and juvenile onset ALS seen in people younger than 25 years old. Men appear to be at a slightly higher risk with a ratio of 1.5:1 compared to women. Median age of onset is 64 years with approximately 5% of affected individuals being less than 30 years old. Median survival time is affected by site of onset with median survival of 2 to 3 years with bulbar onset versus 3 to 5 years for spinal cord onset. Death is secondary to respiratory muscle paralysis and asphyxiation.

The D90A phenotype varies somewhat from the majority of sporadic and familial ALS cases. It has a mean onset of 43 years and a much slower course of progression with a median survival time of 11.5 years post onset. There is no sex predilection for this subset of ALS. Clinical signs begin in the lower extremities as a mix of both UMN
and LMN dominated by UMN spasticity.\textsuperscript{53} As the disease progresses, the LMN disease component dominates over the UMN signs.

DM-affected dogs initially manifest UMN signs in the pelvic limbs that progress to involve the thoracic limbs and later manifest LMN signs.\textsuperscript{2-3} The overall prevalence of DM in the canine population is 0.19\%.\textsuperscript{3,54} There is no sex predilection and a majority of dogs are greater than five years old at the time of onset. Large breed dogs such as the GSDs and Boxers have a reported mean age of onset of 9 years.\textsuperscript{1,54} Pembroke Welsh Corgis have a later onset with a mean age of 11 years.\textsuperscript{3} Initial clinical signs are characterized by general proprioceptive ataxia and asymmetrical UMN spastic paraparesis.\textsuperscript{1,3-4,10,54} Muscle tone and segmental spinal reflexes are initially maintained. Clinical progression to euthanasia averages 6 to 9 months and coincides to when a majority of dogs become non-ambulatory UMN paraparetic.\textsuperscript{1,10} If the affected dog is not euthanized and provided supportive care, clinical signs evolve into a flaccid LMN paraparesis or paraplegia with loss of muscle tone and muscle mass occurring between nine and 18 months post onset. Between 14 and 24 months, the cervical spinal cord will be affected leading to thoracic limb paresis with concurrent paraplegia. Generalized pelvic limb muscle atrophy and urinary and fecal incontinence will also occur at this stage. End-stage disease manifests as flaccid tetraplegia, dysphagia, dysphonia, and respiratory difficulty as both the LMNs in the spinal cord and brainstem become affected.\textsuperscript{2-3,36,54}

Unlike ALS, the natural progression of DM has not been well established since a majority of dogs are euthanized six to nine months from onset as they become non-ambulatory paraparetic. Two reports involving a miniature Poodle and a series of
Pembroke Welsh Corgis described advanced DM signs and survival times of up to 37 months.\textsuperscript{3-4,36,55-56} Because dogs are euthanized at various disease stages, they may help elucidate mechanisms of neurodegeneration earlier in the disease course.

V. Histopathology Comparison

Definitive diagnosis of DM is still based on histopathologic examination. Degenerative myelopathy was described by Averill in 1973 as a degeneration of spinal cord white matter in GSDs.\textsuperscript{1} The classic histologic changes reported are axonal and myelin degeneration that occur in all spinal cord funiculi, but are consistently most severe in the dorsal portion of the lateral funiculi within the middle to lower thoracic region.\textsuperscript{3,11,57} With chronicity, the cervical and lumbar spinal cord become involved.\textsuperscript{4} Non-inflammatory degeneration and neuronal fiber loss of the ascending somatic and proprioceptive sensory tracts and descending motor tracts are typical.\textsuperscript{10,58} The axonal loss and demyelination is replaced by large areas of astrogliosis, or sclerosis, similar to what is seen in UMN-onset ALS.\textsuperscript{59}

Degenerative myelopathy has been described as primary central axonopathy restricted to the spinal cord.\textsuperscript{1,3} Axon and myelin degeneration of the spinal cord occurs in all funiculi and involves the somatic sensory, GP sensory, and motor tracts in the absence of observable neuronal cell body degeneration or loss. Hence the lesion description is best denoted as a segmental degeneration of the axon and associated myelin rather than Wallerian degeneration. In the purest sense, Wallerian degeneration is defined as fragmentation and dissolution of the part of the axon distal to the primary injury of the axon and active digestion and removal of the collapsed myelin by macrophages.\textsuperscript{60} The
pathology of DM involves segments of the axons within the various tracts which would be consistent with either a defect in cells supporting axon maintenance (astrocytes and/or oligodendrocytes) or defects in both anterograde and retrograde axoplasmic transport.\textsuperscript{4} The paucity of spheroids does not support DM’s categorization as a neuroaxonal dystrophy.

Characterization of the brain pathology of DM affected dogs has been limited. Johnston described abnormalities in the red nucleus and lateral vestibular nucleus of the brainstem and in the lateral (dentate) and fastigial nucleus of the cerebellum in DM-affected dogs.\textsuperscript{57} Ultrastructural examination of the red nucleus revealed neurofilament accumulations. Others who examined brains from DM-affected dogs by light microscopy did not find lesions in the brain.\textsuperscript{1,4,11}

The recent discovery of a mutation in the \textit{SOD1} gene provided a better understanding of the clinical spectrum of DM, which is more akin to ALS.\textsuperscript{2} Hallmarks of human ALS include phosphorylated neurofilament accumulation in the proximal axon and neuronal cell body, ubiquinated cytoplasmic inclusions, motor neuron loss, and abnormalities of axonal transport.\textsuperscript{61-62} Degeneration of the motor neurons involves not only the spinal cord but the primary motor cortex as well as the brainstem. \textit{Lateral sclerosis} alludes to the corticospinal tract degeneration and gliosis observed in the anterior and lateral funiculi.\textsuperscript{52} Because the lower motor neuron also degenerates, secondary peripheral nerve system lesions manifest as denervation atrophy, or \textit{amyotrophy}.\textsuperscript{59} Other common pathologies shared amongst the types of ALS in humans include aggregation of misfolded protein, altered RNA metabolism and abnormalities of axonal transport.\textsuperscript{61-62}
Because ALS affects the UMN and LMN systems, this has led to further clinical studies of the peripheral nervous system in DM-affected dogs.\textsuperscript{2,63} Aggregates that bind anti-SOD1 antibody have been detected in neurons of DM-affected dogs.\textsuperscript{2} These SOD1-positive cytoplasmic aggregates are a hallmark finding of SOD1-associated ALS in humans.\textsuperscript{5} Normal age-matched control dogs with wild-type homozygous allele had no SOD1-positive staining of their spinal cord neurons. Interestingly, some asymptomatic heterozygous carriers had lightly staining SOD1 aggregates that may reflect subclinical disease. To date, no distinct light microscopic lesions have been identified in the motor neuron cell body of DM-affected dogs. Neuronal morphometry studies are currently underway to determine whether quantitative changes are occurring. Muscle and nerve pathology appears to vary with disease stage.\textsuperscript{63} Peroneal nerve fiber loss secondary to axonal degeneration, endoneurial fibrosis, and secondary demyelination were demonstrated to occur in dogs with advanced DM.\textsuperscript{2} Muscle specimens also showed excessive variability in myofiber size, typical of end-stage denervation atrophy.

In summary, canine DM may be most accurately classified as a \textit{multisystem central and peripheral axonopathy}. 
I. Transgenic Rodent Models

An understanding of ALS pathophysiology has been severely limited by the paucity of biological material from affected individuals in the early stages of the disease.\textsuperscript{5} There are no previous reports of spontaneously occurring animal models of ALS. Thus, ALS research has relied heavily on transgenic rodent models. Since the 1993 discovery of the first gene mutations associated with ALS, the SOD1 subset, numerous transgenic rodents have been developed.\textsuperscript{46} There are five major categories of transgenic mouse models used in current research settings: SOD1 mutants, TDP43 mutants, intermediate filament disorganization mutants, ALS2 knock-outs, and microtubule transport defect mutants.\textsuperscript{64}

A majority of the insight gained into ALS pathogenesis to date has been a result of the various SOD1 transgenic mice developed. The SOD1 gene encodes the protein copper-zinc superoxide dismutase 1, one of three superoxide dismutase family proteins.\textsuperscript{65} The other dismutases, manganese superoxide dismutase and extracellular superoxide dismutase, are encoded by the genes SOD2 and SOD3, respectively. Superoxide dismutase family members remove superoxide anion radicals that occur secondary to insults such as ionizing radiation as well as electron transport chain by-products of mitochondrial oxygen metabolism.\textsuperscript{65} The SOD1 protein normally is found in the mitochondrial membrane as well as cytoplasmic membrane.
Initially, it was theorized that mutations in *SOD1* decreased free radical scavenging activity leading to peroxidation of neuronal membranes and cell death. Various *SOD1* transgenic mice were developed to test this theory. Currently, there are at least 15 different human *SOD1* mutations engineered into mice. The *SOD1* knockout mice, surprisingly, do not develop ALS clinical signs. They do, however, develop abnormalities such as noise-induced deafness and an age-related peripheral axonopathy. These non-ALS signs may be due to other enzymatic functions of copper-zinc superoxide dismutase. Mice generated to over-express normal human *SOD1* protein have demonstrated axonal loss and motor neuron degeneration in adult animals. These mice, however, never succumb to symptomatic ALS. Clinical signs comparable to motor neuron disease have been seen in the mice expressing the *SOD1* mutations (*hSOD1*\textsuperscript{m}). Signs begin with hindlimb tremors and paresis that progresses to paraplegia before ascending to involve the thoracic limbs. Rate of disease progression in the *hSOD1*\textsuperscript{m} mice is inversely proportional to the gene dosage expressed. The combined studies have strongly supported the notion that the neurodegeneration in *hSOD1*\textsuperscript{m} mice and human ALS is due to a toxic gain of function rather than a loss of function in *SOD1*.

Although the nature of the toxicity is unclear, several experiments suggest the alteration of amino acid sequences destabilizes protein conformation and leads to protein misfolding. Missense mutations can occur at a variety of sites including the active site, within the β strands, or the connecting loops of the *SOD1* protein. Depending on the site of the mutation, the mutant *SOD1* may disrupt the enzyme’s structure by causing isoform destabilization, altered copper or zinc binding, or changing the overall particle
The canine SOD1 E40K mutation is found within the connecting loop. The position has been shown to reduce the net negative charge of the protein. This change in particle charge may lead to protein aggregation secondary to decreased repulsive forces. It is theorized that the aggregates may concurrently overwhelm the protein folding chaperones and the ubiquitin proteosome pathways.

The pathogenesis of motor neuron degeneration in ALS is likely to consist of synergistic interactions between various neuronal cellular mechanisms and supporting glial cells. Chimeric mice with both wild-type and mutant SOD1 genes in different cell populations were developed and revealed the role of glial cells in ALS. If the mutation is only expressed in neurons, neurodegeneration is either delayed or abolished. If both neurons and glial cells express the mutant SOD1 protein, then symptoms develop and progress as predicted. It is now believed that pathology begins within the motor neuron, but it is the glia that amplify toxicity and steer progression of pathology.

It has also been shown that SOD1 mutant mice have disturbances in glutaminergic transmission. Moreover, these mutants have astrocytic dysfunction that correlates with elevated extracellular levels of glutamate. It has been theorized that it is glutaminergic excitotoxicity that leads to motor neuron death.

More recently, studies have revealed a potential secretory pathway secondary to interactions between chromogranins, the Golgi apparatus, and mutant SOD1 protein aggregates. The role of chromogranins, constituents of secretory vesicles found in both neurons and endocrine cells, remains unknown. Because it has been found in the motor endplates of skeletal muscle, it may play a role in release of neurotransmitter.
Urushitani and colleagues demonstrated via *in vitro* studies that mutant *SOD1* protein, in conjunction with chromogranin A (CgA), activates microglia to produce TNF-α. Wild-type *SOD1* did not lead to production of inflammatory mediators. Although the combination of CgA and mutant *SOD1* products causes microgliosis, it is only extracellular mutant *SOD1* gene product that leads to significant neuronal death. It remains to be seen whether these pathways occur in human *SOD1*-associated ALS.

Non-*SOD1* mutations have also been discovered within the last 15 years and led to other transgenic models further adding insight into the complex pathogenesis of ALS. Mutations on chromosome 2q33 in the *ALS2* gene have been linked to the autosomal recessive form of juvenile onset ALS, primary lateral sclerosis, and ascending hereditary spastic paralysis. Nullizygous models of these mutations lead to age-dependent oxidative stress in both motor neurons and Purkinje cells. Alsin, the gene product, is an activator of small GTPases belonging to the Ras superfamily.

Microtubule-based transport is an essential mechanism in axons. ATP-powered protein complexes from the kinesin and dynein families transport proteins from the soma to the axon terminal and vice versa along a microtubule network that is stabilized by the tau protein. Heterozygous transgenic models of the kinesin *KIF1B* gene have demonstrated neurodegeneration via a defect in the transporting synaptic vesicle precursor. These animals develop signs similar to the neuropathy Charcot-Marie-Tooth disease type 2a. Transgenic models over-expressing dynamitin, one of the proteins in the dynein complex, have manifested signs typical of late-onset ALS. Other variant forms of ALS, including fronto-temporal dementia ALS (ALS/FTD), have also had loci identified on chromosome 17q21, specifically in the *MAPT* gene encoding for tau. Some
of the tau transgenic models have demonstrated tau hyperphosphorylation and accumulation of inclusions within motor neurons.\textsuperscript{91-93}

Further insight into neuronal and cytoplasmic inclusions seen in ALS has been provided by the discovery of 30 different mutations in the trans-activating response element (TAR) DNA binding protein (\textit{TARDBP-43}) gene. Such mutations lead to hyperphosphorylation and ubiquination of the \textit{TDP-43} protein that is now recognized as a major component of ALS inclusions.\textsuperscript{94-95} These dominant mutations account for 3\% of FALS and 1.5\% of SALS.\textsuperscript{64} Homozygous knockout transgenic mice die in utero. Heterozygous mice develop mild paresis but histologically do not show neurodegeneration. Mice over-expressing some of the mutations have a dose dependent clinical course. None of the \textit{TDP} mutants that have been created, however, have demonstrated the hallmark cytoplasmic aggregates seen in ALS.\textsuperscript{64}

\textbf{II. Translational Gaps}

Animal models permit the testing of hypotheses regarding pathogenesis of disease and the evaluation of the safety and efficacy of therapeutic interventions. The benefit of the transgenic rodent models in the elucidation of the pathogenesis of ALS cannot be questioned. Unfortunately, for as much as has been learned from mice, the exact mechanism of disease remains a mystery. In the simplest sense, how does protein misfolding lead to a toxic death cascade? The pathogenesis is likely to be multifactorial involving excitotoxicity, impaired axonal transport, neurofilament and protein aggregation, inflammatory mediators, and other as yet undiscovered factors.\textsuperscript{61-62}
In addition, an artificially created disease may differ from spontaneous disease in several respects. Many of the current ALS models utilize transgenic animals with 5 to 15 fold over-expression of mutations of interest. Can such artificially induced models truly correlate to human pathophysiology, especially in light of some of the mutants lacking the hallmark inclusions of ALS? There have been over 150 pharmacologic agents evaluated in transgenic models. Tested therapeutics have ranged from anti-oxidants, anti-aggregate, anti-glutaminergic, anti-inflammatory, and anti-apoptotic drugs. A majority of the agents have not shown benefit in rodents and have been discarded prior to evaluation in human patients. The agents that have appeared promising in the transgenic models and moved into human trials have, unfortunately, failed to be efficacious.

There are many reasons for the failure to translate from transgenic rodents to humans. Firstly, transgenic mice are artificially produced, and those that have been shown to mimic clinical signs of ALS rely on marked gene over-expression. Although clinical signs are similar to ALS, progression of disease in rodents is often disproportionately faster than is seen in humans. This might lead to statistically significant therapeutic results in the mice that would not necessarily occur in humans whose SOD1 burden is not as severe. In addition, SOD1 mutations only account for 20% of FALS and 6% of SALS. It is possible that the mechanisms of disease in SOD1 ALS vary greatly from non-SOD1 ALS. Agents that might show promise against mutant SOD1 might not be successful for all ALS variants.

Additional differences between rodent and human trials involve drug pharmacokinetics, pharmacodynamics, and time of drug intervention. Most notable is the
concept of preclinical and clinical intervention. Often, preclinical therapies are tested in rodents prior to the symptomatic phase of disease. The practical application of data regarding improvement in mice receiving pharmacologic agents prior to the onset of clinical signs or evidence of electrophysiologic abnormalities is questionable since preclinical intervention in humans is highly unlikely. In addition, rodent models have small primitive nervous systems and limited cognitive capacity which may not compare in size and complexity to that of humans.

III. Canine Models

Canine models of disease serve as an ideal intermediate between rodents and humans in the translation of pathophysiology and treatment strategies involving heritable diseases. The value of canine models for heritable disease research has been recognized by the National Institutes of Health through an investment of over $40 million in the canine genome project. Over 300 pure dog breeds are currently registered worldwide.\textsuperscript{38} Most of these breeds have emerged in just the last 300 years, a relatively short evolutionary time course.\textsuperscript{100} The most recent genetic bottlenecks have occurred within the last 100 years. For example, at the end of World War I, only five Leonburger dogs remained in Europe; it is these 5 dogs that sired the majority of the Leonburger dogs alive today.\textsuperscript{100} Selective stud breeding as well as advances in technology and transportation have also further reduced the genetic diversity of dogs. In the United States in 2003, approximately 154 dog breeds were registered, with the top 20 breeds accounting for 70\% of registered dogs.\textsuperscript{101}
Over 450 genetic disorders have been described in dogs, the largest number identified in a non-human species.\textsuperscript{102} Approximately 70\% of the identified segregation patterns have been identified as autosomal recessive, X-linked, or having complex incomplete patterns of inheritance.\textsuperscript{102-103}

A canine model begins with the recognition of clinical signs that mimic the major signs and histopathologic changes found in the human counterpart.\textsuperscript{5} About 360 of the canine heritable diseases are analogous to human diseases.\textsuperscript{102,104} Once identified, the spontaneous canine model may serve to further advance knowledge pertaining to mechanisms of disease, development of diagnostic tools, as well as therapeutic interventions.\textsuperscript{96} This has proven a successful strategy in the evaluation and development of chemotherapy agents targeting various cancers.\textsuperscript{6}

IV. DM as Model of ALS

Before the discovery of the $SOD1$ mutation in DM-affected dogs, there were no previously reported spontaneously occurring animal models of ALS. Dogs have a spontaneous mutation, as opposed to transgenic models that represent with phenotypes induced by over-expression of genes.\textsuperscript{2,64} With natural progression of disease, therapeutic trials would most likely mimic humans in that clinical intervention would not begin prior to the onset of signs. In addition, the dog has a brain and spinal cord that more closely approximates the size of the CNS in humans.

In order to establish DM as an animal model of ALS, objective and quantifiable biomarkers must first be ascertained. Biomarkers are essential in establishing a diagnosis, determining prognosis, evaluating mechanism of disease, and monitoring the
effectiveness of therapeutic agents. A recent review article listed the following as ideal features of an ALS biomarker: high sensitivity and specificity prior to the onset of overt muscle atrophy and weakness, reliably differentiate phenotypes in the early stages, predict patterns of disease progression, change in a conventional manner indicative of progression of disease or response to therapeutic(s), and be affordable as well as practical for both clinicians and patients.\textsuperscript{7} Various biologic changes have been described from the analysis of body fluids, neuro-imaging and neurophysiologic studies of ALS patients.\textsuperscript{7,105} Since loss of the motor unit is the dominant cause of progressive weakness in ALS, a quantitative measure of the motor neuron system, specifically the LMN, is the logical choice for DM evaluation.
CHAPTER 4

GENERAL SOMATIC EFFERENT SYSTEM

The general somatic efferent (GSE) system links the central nervous system (CNS) with the skeletal musculature. In combination, the pyramidal and extrapyramidal systems are responsible for the initiation of voluntary movement and maintenance of postural tone.\textsuperscript{106-109} The UMN influences the LMN, the efferent neuron of the central nervous system, that synapses on skeletal muscles and initiates muscular contraction.\textsuperscript{110} The UMN system also plays a role in controlling and modulating smooth and cardiac muscle activity in the general visceral efferent (GVE) system. The GVE system, however, will not be discussed in this manuscript.

I. Upper Motor Neuron System

The upper motor neuron (UMN) is the portion of the GSE that is confined to the CNS. The UMN component is classically divided into the pyramidal and extrapyramidal systems.

i. Pyramidal System

The pyramidal system involves neurons whose dendrites and cell bodies are in the cerebral cortex. Their axons, either corticospinal or corticonuclear fibers, terminate in either the spinal cord or brainstem gray matter, respectively.\textsuperscript{108} The extensiveness of the pyramidal system varies according to the species. As a general rule, it is more developed in species that exhibit more well-developed fine motor abilities, most notably in the
digits. Primates and humans have the most developed pyramidal systems followed by carnivores.\textsuperscript{106-108} These areas are not as well-defined in animals as they are in humans.

In carnivores, the first order neuron of the pyramidal system is located in the motor area in either the post cruciate gyrus or rostral supersylvian gyrus of the frontal lobes. The gyri are further organized based on the body region the neuron ultimately influences; this is termed \textit{somatotopic organization} and is proportional to the importance of each region’s function.\textsuperscript{106} The motor area of the post cruciate gyrus, for example, is associated with the appendicular muscles whereas the rostral supersylvian gyrus is subdivided into regions responsible for muscles of the head and neck.

The axons of the first-order neuron exit the motor cortex and descend via the corona radiata, the centrum semiovale, and the internal capsule. The axons then travel through the crus cerebri of the ventral mesencephalon before becoming the longitudinal fibers of the pons. In the caudal brainstem, these axons form the \textit{pyramid}. As they continue to descend through the myelencephalon, a majority of the axons will decussate and move dorsally to the lateral funiculus where they form the lateral corticospinal tract.\textsuperscript{108} This tract is somatotopically organized with the striated muscles of the forelimb represented medially, the trunk in the center, and the pelvic limbs laterally. The lateral corticospinal tract in dogs terminates along the entire length of the spinal cord with approximately 50\% terminating in the cervical segments.\textsuperscript{106} Approximately 25\% of pyramidal fibers do not decussate at the level of the pyramids and continue within the spinal cord as the ventral corticospinal tracts. These fibers decussate near their termination sites. Dogs lack a ventral corticospinal tract; whereas in cats the ventral corticospinal tract terminates in the cervical spinal cord.\textsuperscript{106}
Not all of the pyramidal fibers terminate within the spinal cord. As the axons descend through the mesencephalon, metencephalon, and myelencephalon, neuronal fibers decussate to synapse on contra-lateral cranial nerve motor nuclei. These corticonuclear fibers will terminate on the dendrites of motor nuclei of III, IV, V, VI, VII, IX, X, XI, and XII. Although technically these neuronal fibers diverge prior to reaching the pyramids in the medulla, they are still considered a part of the pyramidal system as they initiate voluntary eye, jaw, tongue, pharyngeal, laryngeal, and facial movements.

The second-order pyramidal neurons are interneurons within the brainstem or spinal cord. Within the spinal cord, the first-order (UMN) neuron will synapse on an interneuron located at the base of the dorsal gray horn. This short interneuron synapses on the third-order neuron of the GSE system, the LMN. The corticonuclear neuronal fibers, likewise, synapse on interneurons located in contralateral cranial nerve nuclei. These interneurons synapse on dendrites of cranial nerves responsible for voluntary motor function.

ii. Extrapyramidal System

The second component of the UMN system is the extrapyramidal system. This system encompasses all of the descending motor pathways with the exceptions of the corticospinal and corticonuclear fibers. The extrapyramidal system is a complex multisynaptic circuit with the telencephalic and diencephalic neurons projecting onto the “motor command centers” in the mesencephalon and rhombencephalon. It is
only the neurons located in the lower command centers that ultimately have synapses within the spinal cord.

1. Telencephalic and Diencephalic Centers

The cortical extrapyramidal system neurons are located throughout the cerebral cortex with a high concentration in the motor cortex of the frontal and adjacent parietal lobes. These axons will descend to synapse on lower extrapyramidal nuclei. The basal nuclei, a subcortical collection of neurons, are one of the major relays for the extrapyramidal cortical neurons.

The extrapyramidal basal nuclei include the caudate, accumbens, globus pallidus, putamen, and claustrum. Two other basal nuclei, the septal nuclei and amygdala, are not considered part of the extrapyramidal system but rather function as part of the limbic system. The putamen and pallidum comprise the lentiform nucleus. It is the caudate nucleus that receives the majority of the input from the ipsilateral cortical extrapyramidal neurons. The caudate nucleus also receives input from the substantia nigra, a mesencephalic extrapyramidal nucleus. Efferents of the caudate nuclei project to the adjacent globus pallidus. It is within the globus pallidus that efferents from the other basal nuclei converge and are integrated. The globus pallidus efferent fibers terminate on the ventral rostral thalamic nuclei of which fibers then project back to the motor cortex. This complex circuit serves to modify the initiation of voluntary movement. Although the pathways and circuits between the basal nuclei and the motor cortex are not completely understood, the basal nuclei are considered to be facilitatory in the control of complex patterns of locomotion.
The extrapyramidal nuclei located in the diencephalon include the endopeduncular nuclei, zona incerta, and subthalamic nuclei. Afferents to these nuclei arise from both the cortical neurons and globus pallidus. Efferents project to the thalamic nuclei as well as the mesencephalic and medullary reticular formation.\textsuperscript{106,109}

2. Mesencephalic Centers

In the mesencephalon, the red nucleus and the substantia nigra make up the extrapyramidal mesencephalic reticular formation. Ipsilateral cortical neurons project onto the substantia nigra. The substantia nigra projection axons terminate rostrally on the ipsilateral caudate nucleus to influence the feedback circuit to the motor cortex. The substantia nigra is considered an inhibitory component of the extrapyramidal system and dampens activity of the basal ganglia.\textsuperscript{107}

The red nuclei are located within the tegmentum of the mesencephalon. Input to these nuclei is from the ipsilateral motor cortex. Its axons will decussate upon leaving the nucleus to descend along the contralateral medulla as the rubrospinal tract. Within the spinal cord, the rubrospinal tract is located within the lateral funiculus just ventrolateral to the lateral corticospinal tract.\textsuperscript{109} The axons terminate along the entire length of the spinal cord on interneurons in the ventral horn. These interneurons will then synapse on GSE LMNs. The rubrospinal tract is facilitatory to LMNs that innervate flexor muscles of the contralateral limb. It is the flexor muscles that are responsible for the protraction phase of locomotion.\textsuperscript{106,111} The red nucleus also gives rise to the rubronuclear tract that synapses on contralateral motor nuclei of cranial nerves in the medulla.
The tectum is comprised of the rostral and caudal colliculi. The colliculi are reflex centers for the visual and auditory systems, respectively. The rostral colliculus receives input from the optic nerve while the caudal colliculus receives input from the cochlear nucleus. The efferents from the colliculi decussate in the mesencephalon and descend as the tectospinal tract in the ventral funiculi of the spinal cord. They terminate in the cervical spinal cord on GSE LMNs that innervate neck muscles. The tectonuclear tract terminates on motor nuclei of cranial nerves primarily innervating extra-ocular muscles.

3. Rhombencephalic Centers

Two extrapyramidal centers are found within the rhombencephalon: the pontine and medullary reticular formations. The pontine reticular formation has major facilitatory influence on LMNs innervating extensor muscles, thus maintaining posture during standing.\textsuperscript{109} The pontine reticulospinal tract is located in the ventral funiculus. The medial medullary reticular formation has inhibitory effects on the LMNs innervating extensors muscles. The medullary reticulospinal tract is located in the lateral funiculus. Both of these nuclei receive input from the contralateral motor cortex via corticoreticular fibers that decussate just rostral to terminating on the reticular formation.\textsuperscript{106,109} Fibers of the lateral medullary reticular formation terminate on the medial medullary reticular formation. It is indirectly facilitatory to the extensor muscles by inhibiting the medial reticular formation; this is termed disinhibition.\textsuperscript{109}

The olivary nucleus, located in the medulla, receives afferents axons from the higher level extrapyramidal centers, including those in the telencephalon, diencephalon,
and mesencephalon. Its efferents project onto the contralateral cerebellum. The cerebropontocerebellar pathway is the major source of extrapyramidal information to the cerebellum.\textsuperscript{106}

II. Vestibular Nuclei

Although not directly a part of the extrapyramidal or pyramidal systems, the vestibular nuclei influence posture. The larger of the two vestibulospinal tracts, the lateral vestibular spinal tract, arises from the lateral vestibular nuclei and traverses the entire length of the spinal cord. The medial vestibular nuclei fibers will descend and terminate along the cervical spinal cord in the medial longitudinal fasciculus. This tract influences neck musculature to maintain head posture. These descending pathways synapse on ipsilateral interneurons facilitatory to alpha motor neurons of the extensor muscles. Collateral pathways influence interneurons that are inhibitory to ipsilateral antagonistic flexor muscles. This is considered \textit{reciprocal inhibition}, excitation of one muscle group that is associated with inhibition of another muscle group.\textsuperscript{112-113}

III. Cerebellum

Although not a part of the UMN system, the cerebellum plays a crucial role in movement. Its primary function is the regulation, not the initiation, of locomotion. It coordinates and dampens the movements instigated by the UMN system. It receives input from the spinocerebellar, cuneocerebellar, and vestibulocerebellar tracts regarding general proprioception and special proprioception, respectively.\textsuperscript{109,114} Visual and auditory information is received via the tectocerebellar fibers. Input regarding the UMN system is relayed via the cerebropontocerebellar tract. The cerebellum’s functions may
be divided into three anatomical zones: the medial, intermediate, and lateral zones.\textsuperscript{115}

The medial zone that encompasses the vermis and fastigial nuclei regulates postural tone and equilibrium. The intermediate zone includes the paravermal portions of the cerebellar hemispheres and interpositus nuclei; it coordinates agonist and antagonist muscles during skilled movements. The lateral zone includes the lateral hemispheres and lateral (dentate) nuclei. The lateral zone is involved in intricate sequential motor patterns. The lateral zone is much more highly developed in primates than in domestic animals.\textsuperscript{116}

Neurons of the interpositus and lateral cerebellar nuclei project via the rostral cerebellar peduncle to the red nucleus, globus pallidus, and ventro-lateral thalamic nucleus to ultimately influence the UMN system. The entire circuit between the UMN and the cerebellum is called the cerebropontocerebellar-cerebellorubrothalamocortical pathway.\textsuperscript{115}

In summary the UMN system is made up of the pyramidal and extrapyramidal systems with regulation provided by the cerebellum. The two systems are integrated to initiate voluntary movement and maintain postural tone during both standing and movement. Interneurons of both the pyramidal and extrapyramidal systems as well as the vestibular nuclei synapse on the LMN cell bodies.

IV. Lower Motor Neuron System

There are two major categories of LMNs: general somatic efferents (GSE) and general visceral efferents (GVE). This manuscript will focus on the GSE-LMN. There are two types of GSE-LMNs: the skeletomotor and fusimotor neurons.\textsuperscript{113}
i. **Fusimotor neurons**

The fusimotor neuron, also known as the gamma motor neuron, innervates intrafusal myofibers that form muscle spindles. In domestic animals, approximately one-third of the ventral horn neurons are fusimotor.\textsuperscript{113} Its myelinated axon has a smaller diameter (2-8 micrometers) and conducts more slowly than the alpha motor neuron. The gamma motor neurons along with their muscle spindles play a major role in maintaining basic muscle tone.\textsuperscript{110}

1. **Muscle Spindles**

The muscle spindle provides sensory feedback from the myofibers to the spinal cord regarding muscle length, tension, and how quickly the two are changing.\textsuperscript{112} The spindle consists of intrafusal myofibers that are approximately three to ten millimeters long interspersed and attached via glycocalyx proteins to the larger extrafusal myofibers.\textsuperscript{112} Groups of spindles cluster centrally within a muscle belly. In the center lies the nuclear bag region while the outer portion consists of the nuclear chain fibers.\textsuperscript{110}

Two types of gamma neurons innervate the spindle. The first, the gamma plate neuron, terminates on the nuclear bag. This neuron is responsible for the active stretch of the nuclear bag that stimulates the annulospiral receptor dendritic zone, otherwise known as the primary ending.\textsuperscript{112} Annulospiral fibers are type Ia axons that are approximately 17 micrometers in diameter with a conduction velocity of 70-120 m/sec.\textsuperscript{112} The impulse reaches the sensory cell body located within a spinal ganglion and then enters into the spinal cord via a dorsal root. The axons will then pass through the dorsal horn before
synapsing on a ventral horn alpha motor neuron. This reflexive action is termed the \textit{tonic gamma loop mechanism} and is responsible for maintaining tone.\textsuperscript{106}

The second type of fusimotor neuron, the gamma trail neuron, terminates on the polar ends of the nuclear chain fibers. Nerve impulses from the gamma trail neuron leads to contraction of peripheral portions of the intrafusal myofiber. This contraction activates group II sensory neurons that make up the \textit{secondary ending}.\textsuperscript{106} Impulses from the activated type II fibers will synapse on interneurons that inhibit ipsilateral alpha motor neurons innervating extensor muscles while stimulating interneurons that synapse on alpha motor neurons to ipsilateral flexor muscles. This is termed the \textit{phasic gamma loop} and is responsible for the initiation of flexor reflexes in the generation of gait.\textsuperscript{106}

When the muscle is at rest, the annulospiral receptor is below threshold. When the nuclear bag stretches, as in the case of the patellar reflex, the annulospiral fiber will be activated and ultimately lead to the excitation-contraction of the extrafusal vastus and rectus femoris muscles; this is the \textit{stretch reflex}. This may also occur when gravity causes a stretching of the spindle, for example when standing. Gravity will stimulate the sensory fibers to ultimately excite, or continue to excite, the alpha motor neurons that innervate the extensor muscles.\textsuperscript{106,113}

The gamma neurons receive input from the pyramidal and extrapyramidal systems via interneurons. The majority of the corticospinal tracts of the pyramidal system have their ultimate influence over gamma rather than alpha motor neurons. The tectospinal, rubrospinal, and reticulospinal tracts also influence gamma motor neurons.\textsuperscript{113} Stimulation of the gamma motor neurons will lead to contraction and stretching of the
muscle spindles which in turn activate the annulospiral receptor and type II receptors ultimately activating alpha motor neurons.

2. Golgi Tendon Organ

The Golgi tendon organ is a proprioceptive receptor that detects changes in muscle tension rather than muscle length. It is located in the myotendon junction. When activated, this specialized sensory receptor transmits its signal via rapidly conducting type Ib fibers that average 16 micrometers in diameter. Within the spinal cord, the fibers synapse on interneurons that inhibit the alpha motor neuron responsible for detected muscle tension. In this way, the Golgi tendon organ prevents overload of a muscle. The Golgi tendon afferents act antagonistically to the spindle afferents.

ii. Skeletomotor neuron

The skeletomotor neuron, also known as the alpha motor neuron, innervates extrafusal skeletal myofibers. The alpha motor neuron cell bodies are located in the ventral gray horn throughout the entire spinal cord. The ventral gray horn is somatotopically organized such that alpha motor neurons that innervate the axial muscles are located medial to neurons that innervate the appendicular muscles. The gamma motor neuron serves as the principle link between the UMN system and the alpha motor neuron. The alpha motor neuron, however, is the final common pathway of the GSE system.

Once activated, the axon of the alpha motor neuron exits the ventral gray matter in the ventral nerve root. The ventral nerve root will join with its corresponding dorsal root to become a segmental spinal nerve root. The spinal nerve roots become spinal nerves once
they exit the vertebral column at the intervertebral foramina. Spinal nerves will merge with other spinal nerves to form peripheral nerves which innervate specific skeletal muscles. The peripheral nerve is composed of a mixture of proprioceptive, sensory and motor axons. The alpha motor axon is a large myelinated type A alpha fiber with a diameter of 14-20 micrometers.\textsuperscript{112,114}

iii. Neuromuscular Junction

The axonal supply to a skeletal muscle fiber terminates into a number of terminal buttons or end-feet. These end-feet contain many vesicles that contain acetylcholine, the cholinergic neurotransmitter at these junctions. The endings fit into a depression of the motor end plate, a thickened area of the muscle membrane of the junction. Beneath the motor end plate lies the sarcolemma which is convoluted into junctional folds. These convolutions allow for an increase in surface area. The space between the nerve and the muscle membrane is called the synaptic cleft. The structure in its entirety is known as the neuromuscular junction.\textsuperscript{112}

When the action potential reaches the nerve terminal, voltage-gated calcium channels along the neural membrane open leading to an influx of calcium ions.\textsuperscript{117} The calcium ions cause acetylcholine vesicles to move towards the neural membrane. The vesicles fuse with the membrane and release approximately 10,000 molecules of acetylcholine per vesicle via exocytosis.

Acetylcholine binds to a nicotinic acetylcholine receptor on the postsynaptic sarcolemma.\textsuperscript{112} The acetylcholine receptors are gated ion channels composed of five subunits in adult animals: two alpha, one beta, one delta, and one gamma. The binding of two acetylcholine proteins to the alpha subunits causes a conformational change in the
receptor so that it “opens” to allow ions to flow in or out of the myofiber. Positive ions such as sodium, potassium, or calcium can cross through the receptor lumen. Sodium enters the myofiber much faster than the other ions and this leads to a positive potential change from -80 mV. This local depolarization is called the end plate potential. Because this is occurring at multiple receptors along the post synaptic membrane, the end plate potentials summate and cause the generation of an action potential across the myofiber. The action potential spreads bidirectionally over the myofiber surface and eventually reaches the transverse tubules. These tubules are extensions of the cell membrane and span from the surface to deep within the myofiber where calcium is stored within the sarcoplasmic reticulum. The action potential triggers the opening of calcium channels along the reticulum releasing calcium into the cytoplasm. The calcium ions bind to troponin altering the conformation of the tropomyosin along the actin myofilament. Actin interacts with the thick myofilament known as myosin. It is the interaction between myosin and actin that ultimately leads to muscle contraction.\textsuperscript{112,117}

As long as acetylcholine remains in the synaptic cleft, it will continue to activate acetylcholine receptors. Some of the acetylcholine will diffuse out of the synaptic cleft. The majority of acetylcholine, however, is hydrolyzed by the enzyme acetylcholinesterase within the synaptic cleft. One acetylcholinesterase molecule can hydrolyze approximately 25,000 molecules of acetylcholine per second.\textsuperscript{118} Acetylcholine is broken down into acetate and choline that undergo reuptake into the axon terminal. The mitochondria in the nerve terminal provide the necessary ATP for the re-synthesis of acetylcholine from these constituents.\textsuperscript{112}
V. Motor Unit

The smallest element of extrafusal muscle contraction is the *motor unit*. The motor unit is composed of a single alpha motor neuron and its corresponding myofibers. Each alpha motor neuron may innervate anywhere between three and several hundred myofibers. The number of myofibers associated with one motor unit is dependent on the degree of refined motor function required of the muscle. A large postural muscle such as the gastrocnemius performs gross contractions and has hundreds of myofibers per motor unit. On the other end of the spectrum are the extra-ocular muscles that require precision movements and therefore have approximately 10 myofibers per motor unit.

i. Motor Unit Classification

Motor units are classified based on their myofiber type. Myofibers are classified according to the following properties: speed of contraction, metabolic pathway, and fatigability. Dogs and cats have three major categories of myofibers. Type 1 myofibers are aerobic with oxidative metabolism, contract slowly, and are fatigue-resistant. A majority of the muscles responsible for postural tone, such as the biceps femoris, are predominantly type 1. Type 2 myofibers perform anaerobic glycolysis and are fast-contracting. Type 2 fibers are subdivided based on their level of fatigability. Type 2a fibers have some oxidative potential and are fatigue-resistant. Type 2b, on the other hand, fatigue easily and are not present in dogs. Type 2c fibers are transitional myofibers typically found in neonatal skeletal musculature. The third type of myofiber, type 2M, originates from the first branchial arch in carnivores. The muscles of
mastication, including the temporalis, masseter, digastricus, and pterygoid, are the 2M myofibers found in dogs and cats.\textsuperscript{117}

ii. **Motor Unit Activation**

Studies performed in cats demonstrated that individual motor units differ greatly in contraction speed, but each unit’s myofibers resemble one another.\textsuperscript{121} When an alpha motor neuron is activated, all of its associated myofibers will contract simultaneously. All of the motor units to a specific muscle are collectively termed the *motor unit pool*. When the entire motor unit pool is activated, a coordinated contraction of the muscle occurs. The majority of motor unit pools are composed of a mix of different types of motor units. The advantage of muscles composed of varying motor units is that this allows for a range of force output from twitch tension to tetanic contractions.

Force output variability is a testament to the diversity of tasks that skeletal muscle must perform from maintaining posture to sprinting to complex refined eye movements. The modulation of force is due to a combination of two properties unique to motor units: recruitment and rate coding. These two properties work in conjunction to incrementally raise the force of muscle contraction while simultaneously smoothing out the gradation.\textsuperscript{123}

1. **Recruitment**

Recruitment of motor units leads to an increase in the overall contractile strength of the muscle. As more motor units are activated, an incremental step in the force of contraction will occur. Myofiber type plays a role in which motor units are activated early versus late.
Motor unit strength is determined by the number of myofibers in the motor unit and their cross-sectional area as well as force per unit area.\textsuperscript{124} Weaker motor units have been shown to be oxidative with smaller diameter myofibers as well as having fewer myofibers per alpha motor neuron.\textsuperscript{125} Stronger motor units have more myofibers per motor neuron. Studies in cats have demonstrated that the weakest motor units produce a force of 0.5g while the strongest motor units produce forces of approximately 13kg.\textsuperscript{121} The earliest activated motor units are those that produce weaker forces. Type I myofibers are also found to be activated sooner.\textsuperscript{121} Because type I fibers are oxidative and fatigue-resistant, it is logical that they would be activated earlier due to their ability to maintain force for a longer period of time.

Initially the incremental rise in force is small but will become progressively larger as the more powerful units are activated. The exact mechanism of this orderly recruitment, however, has yet to be identified.

2. Rate Coding

The second method of modulating muscle force output is rate coding. Rate coding entails increasing the frequency of myofiber depolarization by increasing the volley of nerve impulses reaching the neuromuscular junction. Each motor unit will be activated at a base frequency. As more demand is placed on the muscle, the activated motor units will increase their firing frequency until a peak firing rate is achieved.\textsuperscript{126}

As with recruitment, the type of myofiber will influence the base and maximum firing frequencies. Slow-twitch fibers, for example, have an initial rate of 5 Hz and may increase to 20-30 Hz.\textsuperscript{127} Fast-twitch fiber firing rates range between 30 and 60 Hz.
By increasing the firing frequency of the activated motor units, the force gradations occurring secondary to recruitment can be smoothed into a linear relationship from twitches to tetanic contractions.\textsuperscript{123}
CHAPTER 5

ELECTRODIAGNOSTICS

The electrical properties of myofibers and neurons are the basis of electrodiagnostics.

I. Electromyography

Electromyography (EMG) tests the electrical activity of skeletal muscle. The synchronous activation of all the myofibers associated with one motor unit is called the motor unit potential (MUP). Recording the change in the sarcolemmal membrane potential as it depolarizes during contraction utilizes either a surface or needle electrode. The appearance of the MUP on EMG is dependent on the spatial relationship between the activated cells and the recording electrode as the impulse approaches, reaches, and passes away from the electrode. EMG may be used to evaluate three properties of skeletal muscle: insertional activity, spontaneous activity, and voluntary activity (MUPs).

i. Insertional Activity

Insertional activity and is caused by the mechanical disruption of the needle as it is inserted into the muscle. It normally causes a crisp burst of high frequency positive and negative spikes lasting only a few hundred milliseconds. It typically has an abrupt onset and termination, although this is dependent on the degree of movement as well as speed of the insertion.
ii. **Spontaneous Activity**

When an animal is anesthetized, myofibers should be relaxed without evidence of contraction. Once a needle electrode has been positioned and the insertional activity has ceased, no electrical activity should occur.

The exception to this rule is at the end plate region. This is due to small amounts of acetylcholine being randomly released into the synaptic cleft. The quanta released are not sufficient enough to cause a propagating myofiber depolarization. If the electrode is near enough an end plate region, however, this subthreshold change in membrane potential, the miniature end-plate potential (MEPP), will be recorded. This appears as a low amplitude undulating waveform. High amplitude intermittent 100-200 Hz spikes, known as end-plate spikes, are the result of a single myofiber firing.

Spikes are often associated with MEPPs. MEPPs and spikes have little clinical value. If MEPPs are seen without evidence of intermittent spikes, this may suggest that acetylcholine release is insufficient to generate depolarization. This type of change might be seen in hyperkalemic periodic paralysis.\(^{128}\)

iii. **Voluntary Electrical Activity**

EMG can also be used to assess movement-induced electrical activity. As stated earlier, when all of the myofibers of a motor unit depolarize, a synchronous discharge occurs and is recorded as an MUP. Only those fibers that are closest to the recording electrode will influence the amplitude of the MUP.\(^{126}\) As the voluntary contraction strength is increased, recruitment will occur and more motor units will become activated.

With the use of greater force, rate coding will begin to occur and identification of the
individual MUPs will become difficult. When the individual MUPs are indistinguishable, it is termed *interference pattern*.

**iv. EMG Abnormalities**

Voluntary EMG for the evaluation of MUPs is not performed in veterinary medicine as animals are non-compliant patients. Insertional activity may be reduced in end-stage denervation atrophy, fibrosis, or periodic paralysis. It may be exaggerated or prolonged in inflammatory myopathies, early denervation atrophy, or myotonic disorders.\(^{126}\) In early disease states, increased insertional activity may manifest as a few extra positive sharp waves; a number that might easily be missed since a set number of wave forms is not established for the technique in either humans or animals.\(^{126}\)

Denervation hypersensitivity involves a 100-fold increase in myofiber sensitivity to acetylcholine and develops within the first two weeks of denervation.\(^{129}\) Denervation leads to the expression of a new sodium channel in the sarcolemmal membrane. The sodium channel is slow to deactivate and leads to an alteration in the membrane potential that lowers the firing threshold.\(^{130}\) This hypersensitivity is thought to be responsible for the spontaneous depolarization of the sarcolemmal membrane in response to the release of small quanta of acetylcholine, fibrillations.

Fibrillations are the spontaneous firing of single myofibers. They often are not manifested until at least one third of the motor units have degenerated.\(^{131}\) Fibrillation potential amplitude decreases as long as progressive muscle atrophy continues. One study in humans illustrated a decrease from 612 \(\mu\)V to less than 100 \(\mu\)V over the course
Fibrillations are not specific to denervation but can also occur with myopathic processes such as muscular dystrophy, dermatomyositis, and polymyositis. A second type of abnormal spontaneous discharge on EMG is positive sharp waves. Positive sharp waves are also representative of single myofiber firing. They often precede the appearance of fibrillations.

Complex repetitive discharges represent the spontaneous firing of groups of myofibers in near synchrony. The waveforms and frequency of these potentials remain uniform and regular. They have an abrupt onset and cessation. They often involve 10 or more distinct potentials over a 1 to 200 ms time frame. Complex repetitive discharges are seen in chronic denervating diseases as well as myopathies.

Fasciculation potentials occur when groups of motor units discharge spontaneously. The generator of the potential is unknown but may involve action potential volleys from abnormalities within the spinal cord ventral gray horn or nerve terminals. Fasciculations have been described in compressive myelopathies, radiculopathies, nerve entrapment, tetany, and hyperthyroidism. Fasciculations, however, may also occur spontaneously in normal motor units. In order to be considered pathologic, fasciculations must be accompanied by fibrillations or positive sharp waves.

II. Nerve Conduction Studies

Nerve conduction studies are used to evaluate the electrical conduction of either motor or sensory nerves. Motor nerve conduction velocity (MNCV) studies stimulate a peripheral nerve at two or more sites along its length with recording of the depolarization at a muscle belly innervated by the nerve. Depending on the stimulus intensity, some or
all of the motor units to a muscle will be activated. Although ideally synchronized, some slight variation will occur due to the conduction velocity of the individual motor axons.

Although primarily used to evaluated conduction velocity, the technique provides additional information regarding amplitude from baseline to negative peak, duration from onset to either negative peak or final return to base line, and latency. Latency evaluates the time from stimulation of the nerve to onset of the motor unit potential. Conduction time is calculated by subtracting nerve activation and neuromuscular transmission from the latency difference at the two different nerve sites.\textsuperscript{136} Motor nerve conduction studies are primarily used to evaluate the axon and myelin sheath. As a general rule, axonopathies result in decreased amplitudes. Demyelinating disorders will slow conduction time.

i. **Physiologic Considerations**

Some physiologic changes to motor nerve conduction studies should be mentioned. Stimulation of proximal nerve sites will elicit potentials with longer durations and lower amplitudes when compared to distal stimulation. This is due to the activation of slower conducting axons that will progressively lag behind the faster conducting axons. This is termed *physiologic temporal dispersion*.

Studies have demonstrated the effect of age and limb length in normal dogs. In dogs, velocities do not reach adult values until at least 6 months to one year of age.\textsuperscript{137} Between 7 and 10 years of age, velocity will decrease by approximately 15\% in normal dogs.\textsuperscript{138} Limb length will also influence conduction velocity in that longer limbs will exhibit slower conduction velocities and smaller CMAP amplitudes with longer
durations. This is due to the tapering of nerve fibers that occurs at set anatomic levels along a limb.\textsuperscript{139}

**ii. Conduction Abnormalities**

Reduced amplitude with a normal latency is typically observed in generalized axonopathies, junctionopathies, or severe myopathies. Examples of each of these disease processes in dogs are polyradiculoneuritis, botulism, and polymyositis, respectively.\textsuperscript{140} Slowed conduction with normal amplitude implies segmental demyelination without conduction block. This may be due to incomplete compressive injuries as observed with neuropraxia. Demyelinating conditions in dogs and cats that might yield similar abnormalities include globoid cell leukodystrophy and diabetic neuropathy, respectively.\textsuperscript{140-141}

If there is severe demyelination of two internodes, this will lead to conduction block. The neural action potential cannot bridge this region, and this will manifest as a greater than 50\% decrease in the proximal CMAP amplitude when compared to the distal segment. Conduction block is also seen in cats with severe diabetic neuropathy.\textsuperscript{141}

CMAP temporal dispersion and polyphasia may occur secondary to demyelination. This is due to the fact that as myelin is lost, the latency difference between the fastest and slowest conducting fibers will be amplified. This leads to desynchronized action potentials arriving at the neuromuscular junctions causing asynchronous motor potentials. The waveform duration will be increased and the waveform will appear polyphasic.
III.  ALS: EMG and Conduction Studies

EMG and MNCV are a part of the diagnostic criteria of ALS known as the El Escorial criteria (EEC). The EEC was established as a consensus guideline in 1990 by the Subcommittee on Motor Neuron Diseases/ Amyotrophic Lateral Sclerosis of the World Federation of Neurology. They were updated in 1998 and nicknamed El Escorial Revisited or Airlie House criteria. The purpose of these guidelines has been to aid in the diagnosis and categorization of patients for entry into both research and clinical trials.

The criteria involve evaluation of clinical signs, electrodiagnostic studies, neuroimaging examination, clinical laboratory results, neuropathologic examination if applicable, and repetition of clinical and electrophysiologic examination findings six months apart. The four body regions evaluated in EEC include brainstem, cervical, thoracic, and lumbosacral spinal cord segments. There are four categories of diagnosis certainty: clinically definite ALS, clinically probable ALS, clinically probable – laboratory supported ALS, and clinically possible ALS.

Electrodiagnostic features of definite primary LMN degeneration involves evidence of reduced recruitment (reduced interference pattern), large MUPs (large amplitude with a wider duration), and fibrillation potentials. Possible primary LMN degeneration involves at least one or more of the following features: polyphasic MUPs, low amplitude CMAP if the disease duration has been greater than 5 years or there is associated atrophy, presence of complex repetitive discharges, CMAP change between distal and proximal sites on MNCV, 30% decrement in MNCV if the amplitude is greater
than 10% of normal, or a 50% decrement in MNCV if the associated amplitude is less than 10% of normal.\textsuperscript{50}

The variability of ALS onset has made early diagnosis in certain subsets of patients problematic when using the above criteria. One study has shown that the median time from onset of clinical signs to diagnosis is approximately 7 months for people with bulbar-onset versus 10 months for limb-onset ALS.\textsuperscript{143} This study also illustrated a more likely delay in diagnosis in people presenting with \textit{clinically possible ALS} criteria. Clinically possible ALS is defined as UMN and LMN signs in one region or UMN signs in two or more regions or LMN signs rostral to UMN signs. This category involves ALS that cannot be supported by diagnostic testing, but the diagnostic results have excluded other non-ALS diseases. Another study identified 44\% of 100 ALS patients that were initially misdiagnosed and treated either medically and/or surgically prior to ALS diagnosis.\textsuperscript{144} This study also illustrated that non-bulbar onset ALS had a mean onset-to-diagnosis period of 16.4 months versus bulbar-onset of 9 months. Such studies raise concerns in regards to both clinical and research aspects of ALS. Is such a delay in diagnosis altering potential therapeutic benefits? Is the EEC too stringent in excluding potential patients in therapeutic trials? Per the EEC, 20\% of ALS patients in one study were excluded from trials.\textsuperscript{143}

Attempting to apply the EEC to DM-affected dogs may also pose similar challenges, especially in light of the EMG limitations in veterinary medicine. Because voluntary EMG cannot be performed in animals, the only parameters that may be evaluated in DM-affected dogs are insertional and spontaneous electrical activity. Studies evaluating spontaneous EMG activity in human ALS patients have demonstrated
that fibrillations may not manifest until at least a third of the motor units have
degenerated, clearly indicating a lag period between the start of pathology and its
diagnosis.¹³¹

IV. Motor Unit Number Estimation

There is no direct electrophysiologic method of assessing the number of motor
units within a muscle. EMG and motor nerve conduction studies provide qualitative
information but lack sensitivity in identifying early changes or measuring disease
progression. Motor unit number estimation (MUNE) is an electrophysiologic technique
used to quantify motor units. MUNE is determined by dividing the CMAP<sub>max</sub> by the
average single motor unit potential (SMUP).¹⁴⁵ Negative peak area or amplitude may be
used to calculate SMUP and CMAP.

\[
\text{MUNE} = \frac{\text{CMAP}}{\text{SMUP}}
\]

MUNE techniques differ in how the average SMUP is obtained. In humans, the
commonly used methods include modified incremental stimulation, multi-point
stimulation, spike triggered averaging, and statistical methodologies.¹⁴⁵-¹⁴⁸

MUNE has demonstrated LMN loss prior to the onset of clinical signs and has
served as an accurate predictor of progression in human ALS.⁷⁰ MUNE has been shown
to change most rapidly and reliably in ALS longitudinal studies.⁷,⁹,¹⁴⁹-¹⁵⁰ Furthermore,
there is a strong correlation between MUNE and other ALS outcome measures such as
the ALS Functional Rating Scale, forced vital capacity, and maximum voluntary
isometric contractions.⁸,¹⁵⁰-¹⁵¹
The techniques previously described in animals include modified incremental stimulation and multiple point stimulation. 70,152-156

i. Multiple Point Stimulation Technique

Multiple point stimulation (MPS) is the most commonly used technique in animal models. Evoked potentials of different thresholds are recorded by stimulating a peripheral nerve along multiple sites.157 Low grade stimuli intensity is applied at each site in order to activate the lowest threshold motor units. Ideally, a total of between 10 and 20 MUPs are obtained and averaged.146,148

One major limitation of MPS is the availability of use for only the distal muscle groups. This is due to the need to stimulate a nerve with a relatively long and superficial course, thereby, allowing for multiple SMUP acquisitions.157 Rodents are uniform in size and this has made standardizing MPS techniques relatively easy. Dogs, however, have breed anatomical diversity that may make standardizing the technique challenging.

ii. Incremental Stimulation Technique

The second technique used in animals, the incremental technique, requires only one point along a nerve to be superficially accessible. Incremental stimulation was the original technique described by McComas.145 The stimulating electrode remains in the same position and applies graded electrical stimuli to a peripheral nerve. Graded stimulation intensity elicits step wise increases in the size of evoked motor responses due to recruitment of additional motor units. Beginning with a subthreshold stimulation, the first all-or-none response observed is considered activation of the first motor unit. The stimulus intensity is incrementally raised until the next quantally different potential is
observed. This second evoked potential is considered the summation of two motor units secondary to recruitment. Graded stimuli are applied until a total of ten incremental evoked potentials are obtained. The individual motor unit potentials are determined and averaged. The supramaximal CMAP is divided by the average SMUP to yield a MUNE value.

The incremental stimulation has been previously used on experimental dogs undergoing transection and re-anastomosis of the recurrent laryngeal nerve. Four adult research dogs had unilateral transection and re-anastomosis of the adductor and abductor branches of the recurrent laryngeal nerve performed under one anesthetic episode. Videolaryngoscopic and EMG studies as well as MUNE were performed prior to transection to act as a baseline for comparison. Videolarygoscopy and MUNE were performed at 6, 12, and 18 weeks post re-anastomosis. MUNE baseline counts for the thyroarytenoid (TA) and posterior cricoarytenoid (PCA) muscles were 139 ± 22.75 and 130.5 ± 34.12, respectively. At 6 weeks, no electrical activity was noted, suggesting loss of motor units. By 12 weeks, however, MUNE for the TA and PCA were 150 ± 55.27 and 142 ± 33.90, respectively. At 18 weeks MUNE continued to correlate closely with baseline values. The electrodiagnostic estimates obtained were found to be consistent with histologic axon counts (range of 143 to 370) found in the distal branches of the recurrent laryngeal nerve reported in a previous study. The authors’ concluded that MUNE correlated well with histologic counts, EMG, and functional recovery.

Based on the previous canine MUNE study results and the technical challenges in standardizing a multiple point stimulation technique in dogs, the modified incremental stimulation MUNE technique was selected to evaluate a chosen canine hindlimb muscle.
CHAPTER 6
EXPERIMENTAL STUDY

I. Experimental Purpose and Hypothesis

Longitudinal studies of ALS in humans and animal models have shown a strong correlation between MUNE and other outcome measures of disease progression. Human SOD1 mutations account for approximately 20% of familial cases and 10% of sporadic cases of ALS. A missense mutation in the canine superoxide dismutase 1 (SOD1) gene has been identified as a risk factor for canine degenerative myelopathy (DM). DM is an adult onset fatal neurodegenerative disease. Initial clinical signs are general proprioceptive ataxia and UMN spastic paraparesis. With chronicity, clinical signs evolve into flaccid tetraparesis and other LMN signs. DM is the first reported spontaneously occurring ALS homologue. DM-affected dogs could be used to investigate processes underlying motor neuron degeneration, evaluate potential therapeutic interventions, and map modifier loci.

To date, there are no established quantitative outcome measures for DM. If the modified incremental stimulation MUNE technique can be reliably established in normal dogs, it may prove useful in monitoring disease progression and the efficacy of therapeutic interventions in DM-affected dogs. The objective of this study was to establish the modified incremental stimulation technique for MUNE in the extensor digitorum brevis muscle (EDB) of normal dogs.
II. Materials and Methods

The University of Missouri Animal Care and Use Committee approved all procedures for this study. All electrophysiologic studies were performed by two investigators (L.V. or S.K.).

i. Animals

Electrophysiologic studies were performed on nine healthy purpose-bred research dogs and eight healthy pet dogs with the owner’s informed consent (Table 1). Ages ranged between 1 and 10 years (mean 4.8 ± 2.5 years). Ten dogs were younger than 7 years old (mean 3.0 ± 1.4 years). 7 dogs were older than or equal to 7 years old (mean 7.4 ± 1.1 years). Weights ranged between 4.9 and 30.5 kilograms (kgs) (mean 15.6 ± 16.0 kgs). Physical and neurologic examinations, complete blood counts, and serum biochemistry profiles were performed on all dogs. The dogs were pre-medicated with dexmedetomidine (7mcg/kg IM), buprenorphine (10mcg/kg IM), and glycopyrrolate (0.01mg/kg IM). Anesthesia was induced with propofol (6mg/kg IV) and maintained with isoflurane with MAC between 1.5% and 3% administered via an endotracheal tube. Intravenous lactated Ringer’s solution was administered (10ml/kg/hr) while under anesthesia. Anesthetic depth was evaluated with eye positioning, rectal temperature, heart rate, respiratory rate, and indirect blood pressure monitoring. Warm-water blankets were used to maintain the rectal temperature above 98°F (36°C).
ii. **Electrophysiologic Studies**

Procedures were performed and recorded using a Cadwell Sierra electrodiagnostic machine (Cadwell Laboratories, Kennewick, WA) with software (Sierra Wave 8.0) to include MUNE determinations.

For the motor nerve conduction and MUNE studies, the deep branch of the sciatic-peroneal nerve via the EDB was evaluated bilaterally. Each dog was placed in lateral recumbency with the upward limb secured to a sandbag. The hair on the dorsolateral surface of the tarsus was removed between the distal metatarsus and the proximal aspect of the fourth and fifth digit. Skin resistance was minimized by with an abrasive skin prepping gel (NuPrep, Weaver and Company, Aurora, CO) followed by isopropyl alcohol.

1. **Electromyography**

Muscle electrical activity (mV) was recorded using a concentric bipolar needle (26 gauge) electrode inserted into the cranial tibialis, gastrocnemius, biceps femoris, semitendinosus, semimembranosus, and gluteal muscles of the pelvic limb. A monopolar ground electrode was placed subcutaneously over the tuber calcaneus.

2. **Motor Point Identification**

The EDB muscle is considered ideal because of its relatively flat, parallel myofibers that are likely to be equidistant from the recording electrode. There is minimal surrounding musculature to cause artifact. Typically, the EDB has a medial, middle, and lateral head. The medial and middle heads are innervated by two branches of the deep
peroneal nerve while the lateral head is innervated by only one branch. For this reason, the lateral head was used for evaluation. A motor point is the location within a muscle that has the highest concentration of end plates and requires the lowest stimulus intensity to elicit the first all-or-none response when stimulated. Typically, the muscle is directly stimulated to identify the motor point. To the authors’ knowledge the motor point of the EDB has not been reported. Rather than stimulate the EDB directly with needle electrodes likely to cause iatrogenic damage, evoked potentials were used to identify the presumed motor point. The technique previously described for other canine muscles was used to obtain an evoked potential of maximal amplitude with the least stimulating intensity.

3. Motor Nerve Conduction Studies

Motor nerve conduction studies were performed on the sciatic-peroneal nerve as previously described. To minimize damage to the EDB from repeated trials, gel surface silver electrodes (Grass Technologies, Warwick, RI) were used for the recording and reference electrodes. The recording electrode was positioned over the dorsal surface of the lateral head of the EDB at the level of the bony lateral prominence of metatarsal bone V. The reference electrode was positioned over the proximal aspect of digit IV. A monopolar ground electrode was subcutaneously placed over the tuber calcaneus. 37mm monopolar needle stimulating electrodes (The Electrode Store, Enumclaw, WA) were positioned at three sites along the sciatic-peroneal nerve: caudal and deep to the greater trochanter of the femur, lateral to the head of the lateral gastrocnemius tendon insertion at the level of the head of the fibula, and deep to the long digital extensor tendon at the level of the tuber calcaneus.
4. Modified Incremental Stimulation Studies

A modified version of McComas’ original incremental stimulation technique was used.\textsuperscript{145} The cathode was inserted caudal to the long digital extensor tendon at the level of the tuber calcaneus to stimulate the deep peroneal nerve branch (Figure 1). The anode was subcutaneously inserted approximately 1 cm proximal to the cathode. The surface recording electrode was positioned over the dorsal surface of the lateral head of the EDB at the level of the bony lateral prominence of metatarsal bone V. The surface reference electrode was placed dorsally over the proximal aspect of digit IV. The monopolar ground electrode was subcutaneously inserted over the tuber calcaneus.

Stimuli were 50µsec monophasic constant current pulses at a repetition rate of 1/second. High and low pass filter settings were 10 and 10,000 Hz, respectively. Standard amplifier gain settings were used for CMAP and evoked potentials. Stimulus intensity was increased until the supramaximal CMAP negative peak area (mV·msec) was identified and recorded (Figure 2A). Baseline was recorded using a subthreshold stimulation. The stimulus intensity was incrementally raised until the first all-or-none response was evoked. If the evoked potential was reproducible at least three times over ten stimulations, it was deemed stable and digitally recorded as the first SMUP negative peak area (mV·msec). Successive small increments were applied until a different appearing response was evoked. Before the second evoked response was accepted and stored, the response was evaluated for reproducibility and stability in the same manner as the initial potential. This process was repeated to elicit a total of 10 evoked potentials (Figures 2B and 2C). The Sierra Wave software\textsuperscript{®} calculated the difference between consecutive evoked potentials to determine the contribution of the newly recruited motor
unit, SMUP (Figure 2D). The SMUPs were evaluated and the average SMUP calculated and divided into the supramaximal CMAP to yield a MUNE value.

Eight research dogs had successive intermittent MUNE trials performed under different anesthetic episodes at least two days apart. These dogs had two to five trials performed per pelvic limb. One research dog had only one trial performed per pelvic limb. This dog was scheduled to undergo successive intermittent MUNE trials, but for unrelated reasons was excluded from the remainder of the study. Eight pet-owned dogs had two consecutive MUNE trials performed per pelvic limb under one anesthetic episode. Consecutive trials involved removal and repositioning of all electrodes between trials.

iii. Statistical Analysis

Raw data were not normally distributed so log transformation was utilized to normalize the data. Means with standard deviations and medians were obtained for CMAP, SMUP, and MUNE. Two-sample Student’s t-test was used to evaluate right and left pelvic limbs as well as age groups. Significance was defined as P < 0.05. Test-retest reproducibility was assessed with intraclass correlation coefficient (ICC). Stata Statistical Analysis software (StataCorp LP, College Station, TX) was used.
III. Results

i. Animals

All 17 dogs had normal physical and neurologic examinations. Complete blood counts and serum biochemistry profiles were within the reference ranges.

ii. EMG and Nerve Conduction Studies

Twelve of 17 dogs had EMG performed and no abnormalities were observed. Fourteen of 17 dogs had motor nerve conduction studies performed on the left sciatic-peroneal nerve, and 12 of 17 dogs had motor nerve conduction studies performed on the right sciatic-peroneal nerve (Table 2). Mean (±SD) conduction velocities were 70.3 ± 14.4 m/sec and 69.2 ± 9.8 m/sec for the right and left sciatic-peroneal nerves, respectively.

iii. MUNE Studies

A total of 87 MUNE trials were performed in 17 dogs (Tables 3, 4, 5, and 6). One dog had one trial performed on the EDB muscle of each limb. One dog had 5 trials performed on each limb. Ten dogs had two trials performed on each limb. Three to four trials per limb were performed in the remaining five dogs.

Means (±SD) for CMAP, SMUP, and MUNE for each dog’s EDB muscle were calculated from the raw data. Pooled means (±SD) and medians for the left and right limbs were calculated (Table 7). The MUNE mean (±SD) and median for the right hindlimb were 48 ± 24 and 49, respectively. The MUNE mean (±SD) and median for the
left hindlimb were 54 ± 18 and 48, respectively. The right and left pelvic limb MUNEs were compared, and no statistically significant difference was noted (P=0.14) (Figure 4). The dogs were divided into two age groups, less than 7 years old (n=10) and greater than or equal to 7 years old (n=7) (Figure 5). The MUNE mean (±SD) and median for the < 7 years old group were 45 ± 20 and 46, respectively. The MUNE mean (±SD) and median for the ≥ 7 years old group were 54 ± 22 and 49, respectively. No statistically significant difference was noted between age groups (P=0.17).

Data for all 17 dogs were pooled. The CMAP for all trials ranged from 1.89 to 9.32 mV·mSec. Mean (±SD) CMAP for the entire pool was 5.03 ± 1.56 mV·mSec. Combined mean (±SD) SMUP was 0.14 ± 0.11 mV·mSec with a range from 0.04 to 0.78 mV·mSec. Mean (± SD) MUNE values for the entire MUNE pool was 51 ± 21 with a range from 8 to 154.

Eight of seventeen dogs had consecutive MUNE trials performed twice on each limb under one anesthetic episode (Table 8). Eight dogs had intermittent MUNE trials performed under multiple anesthetic episodes. Intraclass correlation coefficients for consecutive and intermittent MUNE evaluations were 0.73 and 0.65, respectively.

IV. Discussion

Electrophysiologic studies provide information on severity and progression of LMN diseases. Although EMG and nerve conduction studies are invaluable in demonstrating pathology and neurolocalization, they provide qualitative information with the potential to miss subtle LMN abnormalities. Motor unit number estimation is a
quantifiable technique that has been established as an outcome measure of motor neuron diseases in humans and transgenic animal models. Recent research has demonstrated LMN involvement in the later stages of canine degenerative myelopathy. MUNE has the potential to serve as a method of evaluating and monitoring the progression of LMN loss in DM.

MUNE techniques previously described in animals include modified incremental stimulation and multi-point stimulation. Multi-point stimulation, the most commonly used technique in transgenic rodent models. One major limitation of multi-point is the availability of use for only the distal muscle groups which are innervated by nerves with relatively long and superficial courses, thereby, allowing for multiple SMUP acquisitions. Rodents are uniform in size whereas canine breed anatomical diversity may make standardizing a technique challenging. Conduction velocities in dogs have previously demonstrated that limb length does impact velocities and CMAP amplitude. Modified incremental stimulation, on the other hand, requires only one point along the nerve to be superficially accessible.

To the authors’ knowledge, histologic nerve fiber counts for the canine EDB muscle have not been reported. This is a limitation of the study since the MUNE results obtained could not be compared to an actual anatomical count. Studies in humans, primates, and rats, however, have demonstrated that MUNE techniques only slightly underestimate motor unit counts compared to actual anatomical counts of nerve fiber.
Human MUNE studies have demonstrated a gradual 50% decrease in the number of motor units in healthy individuals between 20 and 70 years of age.\(^{166}\) In healthy dogs, motor nerve conduction velocity begins to decrease at 7 years with an approximately 15% decrease by 10 years of age.\(^{138}\) In order to assess the effect of age on MUNE, dogs in this study were divided into two groups, younger than 7 years (n=10) and equal to or older than 7 years (n=7). There was no statistically significant difference between age groups (P=0.17). Possible explanations for the lack of difference between the two groups include the relatively small number of dogs and the relatively young mean age of the dogs in the older group (7.4 ± 1.1 years). Longitudinal studies in normal dogs ≥ 10 years old is warranted to better evaluate the natural impact of aging on MUNE.

One of the major concerns with MUNE techniques involves reproducibility. There are only a few studies that have reported test-retest reliability in humans.\(^{167-171}\) The statistical method has the highest reliability with correlation coefficients as high as 0.95.\(^{167}\) Modified incremental stimulation in humans has correlation coefficients ranging from 0.64 to 0.85.\(^{168}\) Greater variability in data has been observed when performing intermittent trials when compared to consecutive trials.\(^{145,168}\) Previous studies recommend performing two consecutive trials per session and averaging them to decrease variability.\(^{166,170}\) Fewer reports exist on reproducibility of MUNE in animal species.\(^{155,172}\) Intermittent modified incremental stimulation in rabbits had a correlation coefficient of 0.75 when evaluated 30 days apart.\(^{155}\) In the current study, eight dogs had intermittent successive trials and eight dogs had consecutive trials with correlation coefficients of 0.65 and 0.73, respectively. These numbers closely parallel those in humans and transgenic models.
Other concerns regarding the modified incremental stimulation technique that have been previously raised in both human and transgenic models are based on assumptions that may lead to data overestimation and variability. The first assumption is that the SMUPs obtained at low stimulus intensities are representative of the motor unit population of the entire muscle. Studies have demonstrated that muscles have a small percentage of motor units with large activation thresholds and potentials equaling as much as 30% of maximal CMAP. Muscles are composed of various types of motor units, and the percentage of each type of motor unit in a muscle is dependent on the function of the muscle. If only the smallest SMUPs are evaluated, an erroneously overestimated MUNE will be calculated. In 2001, a symposium on MUNE techniques was held to establish standards, including evaluation of small SMUPs. The consensus was that SMUPs with an area less than 25 μV·msec or amplitude of less than 10 μV would be excluded in order to minimize motor unit overestimation. In the current study, the mean SMUP area for the pooled data was 140 μV·msec ± 110 μV·msec, with the smallest SMUP recorded being 30 μV·msec, in keeping with the standards established in 2001.

Another technique assumption is that different appearing evoked potentials are the result of sequential recruitment of individual motor units. Some motor units, however, have overlapping activation thresholds termed *alternation*. Alternation leads to activation of different combinations of motor units causing the production of dissimilar evoked potentials secondary to phase cancellation. This may lead to an overestimation of MUNE. Since the incremental stimulation technique was first described, it has been modified in order to minimize alternation. One major modification has been to subtract
successive incremental evoked potentials from each other in order to better evaluate SMUPs. An automated protocol to evaluate SMUPs for alternation has also been developed and is available with most standard incremental MUNE software packages. Both of these modifications were applied to the current study.

Another potential source of variability is the subjective assessment of the evoked potentials by the operator. As discussed earlier, one of the assumptions of the technique is that quantally different potentials are due to recruitment of additional motor units. Although guidelines for alternation and minimum SMUP size have been established, observer bias when accepting or rejecting evoked potentials is still possible. In the present study, the intermittent trials were performed by two observers. The consecutive trials were performed by one observer which may have increased the correlation coefficient of these trials. Correlation coefficients for both types of trials, however, were within the ranges seen in both humans and transgenic models. As recommended in human studies, two trials performed per session and averaged may help decrease variability particularly in regards to longitudinal evaluations.

Since its inception in the 1970s, MUNE has been used to evaluate many types of neuromuscular disorders in humans, most notably ALS. Longitudinal studies of ALS have shown a strong correlation between MUNE and other outcome measures, identified LMN loss prior to development of clinical weakness, and accurately predicted duration of survival.\textsuperscript{175-176} MUNE has also been used in several animal models of neuromuscular disorders.\textsuperscript{70,152,154-155} As in humans, the technique has shown motor unit loss in asymptomatic transgenic \textit{SOD1} mutant mice long before clinical weakness manifests.\textsuperscript{70}
The hallmark clinical signs of ALS include UMN and LMN system degeneration and the progressive spread of these signs within a region or to other regions, a pathologic continuum of a multisystem disorder. At disease onset, an ALS patient usually presents with only upper or lower motor neuron signs that involve either limb muscles or muscles innervated by bulbar (brain stem) nuclei. Thus, these forms of ALS are termed UMN onset, LMN onset, or bulbar onset. Degenerative myelopathy is a progressive, adult onset neurodegenerative disease. Initial clinical signs are characterized by general proprioceptive ataxia and UMN spastic paraparesis. Later in the disease course, clinical signs evolve into a flaccid tetraparesis and other LMN signs. Definitive diagnosis of DM is still based on histopathologic examination. Histopathologic changes include degeneration and neuronal fiber loss of ascending sensory and descending motor pathways that are most severe in the mid- to caudal thoracic spinal cord. In dogs with advanced DM, nerve specimens show fiber loss, and muscle specimens have changes typical of denervation atrophy. In summary, canine DM is most accurately classified as a multisystem central and peripheral axonopathy.

Since DM is a spontaneous disease with uniformity in onset of clinical signs and disease progression, dogs affected with DM could be used to investigate processes underlying motor neuron degeneration, evaluate potential therapeutic interventions, and map modifier loci. Moreover, the dog has a brain and spinal cord that more closely approximates the size of a human’s nervous system. Spontaneous canine models offer a ready clinical population for evaluation of therapies in a setting that closely mimics human clinical trials. This has proven a successful strategy in the evaluation and development of chemotherapy agents targeting various cancer types.
Biomarkers are useful in establishing a diagnosis, determining prognosis, evaluating mechanisms of disease, and monitoring the effectiveness of therapeutic trials. To date, there are no established outcome measures for monitoring disease progression of DM. The LMN component of DM has not been thoroughly described or evaluated electrophysiologically. The modified incremental stimulation MUNE technique appears applicable and reproducible in healthy dogs. These results provide normal data for healthy dogs and document the potential utility of EDB modified incremental stimulation MUNE for longitudinal monitoring of lower motor neuron loss in DM affected dogs.
Figure 1. Electrode placement for incremental stimulation of the canine deep peroneal nerve. a) Illustration and b) photograph of one of the subjects with electrodes placed. A, cathode, inserted caudal to the long digital extensor muscle tendon at the level of the tuber calcaneus; B, anode, subcutaneously inserted 1 cm proximal to cathode; C, surface recording electrode, over lateral head of EDB at the level of bony lateral prominence of metatarsal V; D, surface reference electrode, over proximal digit IV; E, ground needle electrode, subcutaneously inserted over tuber calcaneus.
Figure 2: Screen capture of incremental stimulation technique in a dog, (A) with maximal compound muscle action potential, CMAP$_{\text{max}}$, (B) 10 stacked incremental evoked potentials, (C) individual evoked potentials, (D) and single motor unit potentials, SMUPs.
Table 1. Age, gender, and weight of dogs

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Table 3. CMAP and SMUP data for the right hindlimb trials

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*CMAP, compound muscle action potential; SMUP, single motor unit potential*
Table 4. MUNE and temperature data for the right hindlimb trials

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*MUNE, motor unit number estimation*
Table 5. CMAP and SMUP data for the left hindlimb trials

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CMAP, compound muscle action potential; SMUP, single motor unit potential
Table 6. MUNE and temperature data for the left hindlimb trials

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MUNE, motor unit number estimation
Figure 3: Box-whisker plot of individual dogs’ MUNE, with median, 75th and 25th percentiles.
Table 7. CMAP, SMUP, and MUNE means and medians for individual dogs

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Mean ± Standard deviations

|        | 5.02 ± 1.50 | 5.03 ± 1.67 | 0.17 ± 0.14 | 0.12 ± 0.06 | 48 ± 24 | 54 ± 18 |

Median

|        | 5.24 | 4.93 | 0.12 | 0.09 | 49 | 48 |

*Compound muscle action potential, CMAP; single motor unit potential, SMUP; motor unit number estimation, MUNE*
Figure 4: Comparison of right and left pelvic limb MUNE. No statistically significant difference between sides (P=0.14).
Figure 5: Comparison of MUNEs in dogs < 7 years old and dogs ≥ 7 years old. No statistically significant difference between age groups (P=0.17).
**Table 8.** Right and left MUNE intermittent and consecutive trials

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<th>Dog</th>
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*MUNE, motor unit number estimation*
Figure 6: Comparison of consecutive trials in 8 dogs. Intraclass correlation coefficient = 0.73.


64. Swarup V, Julien JP. ALS pathogenesis: Recent insights from genetics and mouse models. Prog Neuropsychopharmacol Biol Psychiatry 2010.


