EFFECT OF EXERCISE ON RETROGRADE TRANSPORT IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

by

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ABSTRACT

The potential for exercise to improve function and delay disease progression in a mouse model of amyotrophic lateral sclerosis (ALS) has been examined in some detail. Recent studies have shown that retrograde transport is diminished throughout disease progression in this mouse model. The finding that exercise plus viral delivery of IGF-1 significantly improves lifespan of the G93A transgenic mouse highlights the need to investigate the mechanisms by which exercise may alter factors associated with the disease. Therefore, G93A and wild-type mice underwent an eight-week treadmill running protocol, and retrograde transport and neuromuscular junction integrity were examined. Retrograde transport was significantly improved ($p<0.001$) by exercise training in the transgenic animals. The percentage of innervated muscle fibers was also improved ($p<0.01$), and this improvement was highly correlated to the improvement in retrograde transport ($r=0.75$). These data indicate that exercise may enhance delivery of neurotrophic factors from skeletal muscle to motor neurons.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, rapidly progressive and fatal neurodegenerative disease affecting motor neurons in the brain and spinal cord. Death of motor neurons eventually leads to complete paralysis and death within 3-5 years of symptom onset. It is a common neurological disease with a worldwide prevalence estimated at 4-6/100,000 of the population with a peak prevalence of 33/100,000 for males and 14/100,000 females in the 60-75 age group (Majoor-Krakauer et al., 2003). Although the etiology is not completely understood, common pathological mechanisms in the different forms of ALS have been identified.

The majority of ALS cases (90%) are sporadic with no known genetic link, however the remaining 10% of cases are known as familial ALS (fALS) which is inherited as an autosomal dominant trait (Gurney et al., 1994). Approximately 20% of fALS cases are attributed to mutations of the SOD1 gene encoding for copper, zinc superoxide dismutase (Rosen et al., 1993). The clinical features of the two forms of ALS are virtual indistinguishable, although age of onset is earlier (45 versus 56 years), and prognosis is poorer (1.5 versus 3 years) in the familial ALS form (Gurney, 2000).

Much of the ALS research to date has attempted to delineate how and why motor neurons die and how to promote their survival. Although the exact cause of motor neuron death is unknown, there is evidence for the involvement of environmental factors, oxidative stress, mitochondrial dysfunction, glutamate excitotoxicity, gliosis,

neurofilamentous aggregation, and apoptosis among others (Robertson et al., 2002; Strong and Rosenfeld, 2003). In familial cases where the gene for mutant SOD1 is identified, pathology involves a toxic gain of function of the Cu/Zn superoxide dismutase enzyme (Gurney, 2000).

Currently the only treatment approved for human trials is riluzole, which prolongs survival by about two months through its action of reducing synaptic glutamate release (Miller et al., 2003). Exciting new research, however, involving the use of neurotrophic factors through gene therapy to prevent motor neuron death has shown promise in various studies on murine models of ALS. Studies suggest that optimal administration of neurotrophic treatments relies on sufficient retrograde transport from muscles to motor neurons (Boillée and Cleveland, 2004). Retrograde transport is likely disrupted before symptom onset in ALS (Fischer et al., 2004), meaning the effectiveness of these new treatments will be diminished by the time the disease is discovered in most ALS patients.

The association between exercise and ALS risk has intrigued researchers and clinicians alike for decades. High profile ALS victims like Lou Gehrig, three members of the San Francisco 49'ers, and boxer Ezzard Charles, along with epidemiological studies linking ALS risk to lifelong physical exercise, manual labour, low body weight, and even playing professional soccer (Scarmeas et al., 2002; Chiò et al., 2005), have fuelled this debate. Although recent studies have been unable to show such associations between exercise and ALS risk, the question remains whether chronic activation of motor units through exercise somehow exacerbates an underlying susceptibility to ALS (Longstreth et al., 1998). Exercise training has been shown by several studies to improve survival in a mouse model of fALS through as yet unknown mechanisms. This thesis sought to

investigate the effects of exercise training on retrograde transport rates and neuromuscular function in the same mouse model as possible explanations for the improvements in survival seen previously.

1.2 Pathways to Motor Neuron Degeneration in ALS

1.2.1 Where Does Motor Neuron Dysfunction Begin?

It is generally accepted that the weakness and death in both ALS patients and the $SOD1^{G93A}$ mouse model of ALS are a direct consequence of the death of motor neurons. Paralysis and severe muscular atrophy are seen towards the end of life in both ALS patients and animal models. It is currently debated, though, whether the motor neurons die resulting in denervation of skeletal muscle leading to atrophy and paralysis, or disease begins at the distal end of axons causing a so-called "dying back" neuropathy. The "dying back" theory involves progressive denervation of skeletal muscles, leading to the degeneration of axons and eventually death of motor neurons. The traditional "dying forward" view is evidenced by the various potential direct causes of motor neuron death at the cell body. In some cases of familial ALS, mutant superoxide dismutase (mSOD1) is involved in the death of motor neurons. So far, over 100 missense mutations have been identified in the gene encoding for SOD1 (Robertson et al., 2002) that account for 20% of fALS cases. The pathology involves a toxic gain of function of mSOD1 rather than a loss of free radical scavenging as once thought (Julien, 2001). The toxic gain of function could involve aberrant copper-mediated catalysis and/or failure of binding to zinc, which could lead to peroxynitrate-induced nitration of tyrosine residues or production of toxic

hydroxyl radicals leading to damage of various cell targets and eventual cell death (Julien, 2001).

Other potential causes of direct motor neuron death include glutamate excitotoxiciy, mitochondrial dysfunction, apoptosis, and neurofilamentous inclusions (Julien, 2001). Glutamate induced excitotoxicity causes alterations in cytosolic calcium homeostasis leading to cell death via calcium-dependent caspases and the protein kinase C (PKC) pathway (Krieger et al., 1996). Evidence of mitochondrial involvement in ALS includes impaired oxidative metabolism leading to cytochrome c release and activation of cell-death caspases (Siciliano et al., 2002), and a massive amount of vacuoles present in mitochondria seen at symptom onset before cell death occurs (Kong and Xu, 1998). These mitochondrial changes are seen in mSOD mice, but could also be involved in sporadic ALS stemming from some environmental stress and free radical damage. Recent evidence suggests that apoptosis is the mechanism of eventual cell death in ALS motor neurons (Guegan and Przedborski, 2003). Neurofilamentous protein aggregations are seen in both motor neuron cell bodies and axons as a pathological hallmark of both sporadic and familial ALS (Julien, 2001). These protein aggregations are thought to be involved in ALS pathology mostly through disruption of axonal transport (Julien et al., 1998).

Evidence for a "dying back" neuropathy in ALS, where pathology begins at the neuromuscular junctions and progresses up axons to motor neurons, has been mounting in the last decade. In $SOD1^{G93A}$ mice, selective loss of fast-firing neuromuscular synapses was observed as early as 50 days of age (Frey et al., 2000), along with loss of functional motor units as early as day 40 (Kennel et al., 1996). This happens well before motor

neuron death, which is typically seen at about 80-90 days of age in this ALS model. This loss of functional synapses was further quantified in a study that found denervation of 40% of motor endplates at day 47 and loss of 60% of ventral root axons at 80 days, before significant motor neuron death at day 100 (Fischer et al., 2004).

1.2.2 Neurofilament Inclusions

Neurofilament (NF) inclusions in the axons of ALS motor neurons could impede axonal transport of neurotrophic factors, organelles, and structural components resulting in the so-called "dying back" neuropathy (Julien, 2001). This abnormal accumulation of neurofilaments is typical of ALS, occurring in about 70% of all cases (Collard et al., 1995). Neurofilaments play an important role in mechanical support of the cell, especially in the structure of the large myelinated axons. One study found that mRNA levels of the small neurofilament subunit (NF-L) are severely decreased in ALS, which could provoke accumulations of the NF-M and NF-H subunits (Wong et al., 2000). Mice over-expressing the NF-H subunit developed severe motor dysfunction without motor neuron death (Beaulieu et al., 2000), but function was rescued when NF-L subunits were co-expressed in a dose dependent fashion (Meier et al., 1999). A recent study used gene replacement to remove phosphorylated tail domains of NF-M and NF-H subunits to prevent intra-axonal crosslinking, resulting in significantly slowed disease progression in mSOD1 mice (Lobsiger et al., 2005).

The length of axons innervating skeletal muscles makes motor neurons especially vulnerable to disruptions in axonal transport. Collard et al. (1995) found that NF-H over expression disrupted slow axonal transport of cytoskeletal elements as well as

mitochondria, suggesting that disturbances in energy metabolism due to lack of organelle transport could contribute to motor neuron death. Studies in this area provide further evidence that axonal transport disturbances in ALS contribute to the disease pathology, and indicate improvements in retrograde transport as potentially therapeutic.

1.2.3 Anterograde and Retrograde Transport Disturbances in ALS

Axonal transport defects are one of the earliest pathological features in mSOD mice, which may even occur during embryonic development (Kieran et al., 2005). The slow component of anterograde axonal flow is disrupted in ALS and this could contribute to motor neuron death (Chou and Norris, 1993). Studies in $SOD1^{G37R}$ and $SOD1^{G85R}$ transgenic mice showed slowing of slow axonal transport of neurofilaments and tubulin six months before disease onset (Williamson and Cleveland, 1999), indicating this is a very early event in the disease process. In addition, axonal swellings observed near end stage in that study showed intense reactivity for neuronal tubulin. Researchers also found increased amounts of neurofilament and mitochondria in the axon hillocks and initial segments of axons of anterior horn cells, indicating impaired fast and slow axonal transport early in disease progression (Sasaki et al., 2005). There is also evidence that a portion of mSOD1 is transported in the slow component of axonal transport and could be responsible for damage to the transport motors as well as damage to tubulin and other transported proteins (Borchelt et al., 1998). These mechanisms could lead to motor neuron death through the simple mechanism of axonal strangulation.

Retrograde transport may also be severely affected in ALS, again tying into the distal axonopathy theory. In a mouse model of a rapid onset motor neuron disease, the

progressive motor neuronopathy (pmn) mouse, retrograde transport rates of the fluorescent neurotracer Fast blue were 46% lower in pmn than in control mice (Sagot et al., 1998). Retrograde transport rates and motor neuron survival were restored in pmn mice with a dominant Wlds ("slow Wallerian degeneration") mutation, which slows axon degeneration and synapse loss (Ferri et al., 2003). Retrograde transport of the *lacZ gene* was shown to be diminished in the mSOD1 mouse model of ALS following injection into the gastrocnemius muscle (Murakami et al., 2001). The velocities for *lacZ* retrograde transport were estimated to be 2.1 and 0.05 mm/24 for non-transgenic and transgenic mice, respectively.

Further support for retrograde transport disturbances in ALS comes from studies involving dynein. Cytoplasmic dynein is the molecular motor responsible for fast retrograde axonal transport. In mice where dynein is inactivated, a progressive motor neuron degenerative disease with characteristics similar to ALS develops with severely disrupted retrograde transport (LaMonte et al., 2002). Two transgenic models with dynein mutations have been developed, the *Legs at odd angles* (*Loa*) and *Cramping* (*Cra*) mice, both with progressive motor neuron degeneration and Lewy-like inclusion bodies resembling human pathology of motor neuron diseases (Hafezparast et al., 2003). Similarly, mutation in the dynactin complex, a dynein activator, inhibits both retrograde and anterograde axonal transport (Martin et al., 1999), and can result in a disorder mimicking human motor neuron disease (Puls et al., 2003). With ALS mouse models showing similar abnormalities in microtubule-based axonal transport, retrograde transport is likely disrupted in ALS as well.

Surprisingly, double heterozygote *Loa*/SOD1 transgenic mice with both disruption in dynein and mutant SOD1 showed delayed disease progression and increased lifespan compared to mSOD1 mice (Kieran et al., 2005). This occurred along with a complete recovery of axonal transport in the double transgenic mice suggesting that impaired axonal transport is a prime cause of motor neuron death in ALS. Similarly, double heterozygote *Cra1*/SOD1 transgenic mice, with a different mutation in the dynein gene, showed delayed disease progression and significant increase in survival time over mSOD1 mice (Teuchert et al., 2006). The authors of these studies are unable to explain this paradoxical improvement in mSOD1 mice, but suggest it may result from returning the balance of retrograde and anterograde axonal transport in the double-heterozygote motor neurons promoting axonal homeostasis (Kieran et al., 2005).

1.2.4 Alterations at the Neuromuscular Junction

As mentioned previously, studies in the mSOD1 mouse have demonstrated degeneration of the neuromuscular junction earlier than loss of motor neurons is observed (Fischer et al., 2004; Frey et al., 2000; Kennel et al., 1996). A case report from an ALS patient who died unexpectedly early showed normal morphology of motor neurons but grouped atrophy and angulated muscles fibers indicating chronic denervation-reinnervation of muscles (Fischer et al., 2004). Grouping by muscle fiber type is thought to occur as a result of fibers reinnervated by collateral sprouts converting to the fiber type of the motor neurons now innervating them (usually slow-twitch type I fibers). These studies suggest that the death of motor neurons could begin through denervation and loss of trophic support from muscle. Mutant SOD1 is also expressed in muscle tissue, but it is

not yet known whether its presence there contributes to disease pathogenesis (Boillée et al., 2006).

Studies have shown mSOD1-mediated motor neuron disease involves predictable and selective vulnerability patterns by subtypes of motor units, axons, and muscle fibers (Frey et al., 2000; Pun et al., 2006). Specifically, fast-fatigable (FF) motor neurons are affected first, fast-fatigue-resistant (FR) motor neurons are affected near the onset of symptoms, and slow (S) motor neurons are resistant to disease. The muscle can be sub-compartmentalized into classes (1, 2, or 3) with very different patterns of denervation (Pun et al., 2006). In class-1 compartments, which are composed almost entirely of FF fibers, all neuromuscular junctions (NMJ) become abruptly and permanently denervated. In class-2 compartments, composed of FF and FR fibers, NMJs are denervated and then become unstably reinnervated only to experience permanent axon loss later on in disease progression. Class-3 compartments are composed of FR and S fibers, and denervation of FR fibers results in collateral sprouting and stable reinnervation by disease resistant (S) motor neurons. Another recent study described two distinct subpopulations of motor units in mSOD1-mediated ALS; "winter tree" motor units showed complete denervation and no signs of growth or reinnervation, and "compensators" showed abnormal enlargement and many thin, reinnervating branches (Schaefer et al., 2005). Identification of factors that protect these compensatory motor units from degeneration may provide new directions for therapeutic interventions.

1.2.5 Apoptosis and ALS

Regardless of the early pathology in motor neuron death, apoptosis is generally accepted as the final stage in the death of motor neurons. Apoptosis, or programmed cell death, in a disease model can be viewed as the disruption of the balance between pro- and anti- apoptotic signals in the cell. Although the morphology of dying neurons in ALS patients is not identical to those in an apoptotic model, expression of apoptotic markers such as DNA fragmentation, cytochrome c release from mitochondria, levels of pro-apoptotic Bax, and cell-death caspases have been observed in human tissue and animal models of ALS (Guegan and Przedborski, 2003).

Treatments with the anti-apoptotic protein Bcl-2 have had positive results, particularly with protection of motor neurons. Overexpression of Bcl-2 in $mSOD1^{G93A}$ mice delayed onset and prolonged survival in one early study (Kostic et al., 1997). However, more recently Bcl-2 treatment via an adeno-associated virus (AAV) was able to increase the number of surviving motor neurons and delay onset of symptoms, but did not prolong survival in the G93A mouse model (Azzouz et al., 2000). Also, pmn mice were crossed with mice overexpressing Bcl-2, which improved motor neuron cell body structure and rescued facial motor neurons but had no effect on survival time or preventing the degeneration of myelinated axons (Sagot et al., 1995). With the potential for physical exercise to decrease apoptosis either directly (Siu et al., 2004) or indirectly through increases in neurotrophic factors (Cotman and Berchtold, 2002), there is potential for its use as a treatment to improve motor neuron survival.

1.3 Potential Treatments of ALS

1.3.1 Neurotrophic Factors and Gene Therapy in the Treatment of ALS

Motor neuron survival is dependent on continual trophic support from muscle fibers, and this support relies on efficient retrograde axonal transport. Increased survival and function of mSOD1 mice was seen after adenoviral-vector administration of glial cell-derived neurotrophic factor (GDNF) (Acsadi et al., 2002). Transforming growth factor-beta-2 (TGF-β2), which is retrogradely transported by motor neurons from the sub-synaptic portions of muscle fibers, was shown to be an important survival factor for motor neurons (Jiang et al., 2000). Exogenous brain-derived neurotrophic factor (BDNF) administration retards motor dysfunction in the wobbler mouse model of motor neuron disease through decreases in denervation, muscle atrophy, and motor axon loss (Ikeda et al., 1995).

Gene therapy using various viral vectors (Azzouz and Mazarakis, 2004; Yamashita et al., 2001) is a promising potential treatment for ALS. One example of this involves insulin-like growth factor-1 (IGF-1), where initial trials showed either no effect or only mild benefits (Boillée and Cleveland, 2004). Recently, however, Kaspar and colleagues (2003) were able to prolong life and delay disease progression in the $mSOD1^{G93A}$ mouse through treatment with adeno-associated virus carrying the gene for IGF-1 (AAV-IGF1). This virus was injected into the medial gastrocnemius then transported retrogradely to motor neurons where IGF-1 could be replicated and released for sustained delivery. An interesting finding of this study is the importance of retrograde delivery, since effects of IGF-1 were reduced when delivered by a lentivirus that is not efficiently transported (Kaspar et al., 2003). Also important was the fact that survival was

still improved when the AAV-IGF1 was delivered after symptom onset since in the majority of human ALS patients treatment will only be possible well after the onset of symptoms (Kaspar et al., 2003). Similar findings were reported following treatment of $mSOD1^{G93A}$ mice with vascular endothelial growth factor (VEGF) delivered with retrogradely transported equine infectious anemia virus (EIAV) (Azzouz et al., 2000).

Another study attempted to classify the neurotrophic effects on motor neuron diseases into those that affect or act via retrograde transport (BDGF, CNTF, and NT-3) and those that act only on the cell body (GDNF, NGF, and Bcl-2), noting that the former are more effective in attenuating disease progression (Sagot et al., 1998). Similarly, significant improvements in mSOD1 mice were seen following treatment with IGF-1 but not with GDNF (Kaspar et al., 2003). Many IGF-1 related improvements are likely related to decreases in apoptosis through the signalling of a cell survival cascade. Downstream in this cascade is Akt, a protein kinase activated through phosphorylation by various growth factors, including IGF-1. This study found 38% higher phosphorylated Akt levels in lumbar spinal cords of AAV-IGF1 treated mSOD1 mice compared to controls (Kaspar et al., 2003). Also, treatment with ciliary neurotrophic factor (CNTF) was shown to alleviate axonal vulnerability and transport defects in both mSOD1 mice (Pun et al., 2006) and pmn mice (Sagot et al., 1998).

1.3.2 Exercise and ALS

The role of exercise in the pathogenesis and treatment of ALS has been controversial. In the United States, ALS is commonly known as Lou Gehrig's disease after the famous baseball player who died of the disease in the 1930's. In addition, three

players of the San Francisco 49ers football team were diagnosed with ALS in the 1980s (Kirkinezos et al., 2003). While these cases are likely to be coincidental, heavy exercise has previously been proposed as a risk factor along with participation in varsity athletics, reduced body fat, and a body mass index under 25 (Scarmeas et al., 2002). A similar result was seen in a study that showed increased risk for ALS and other motor neuron diseases in subjects who reported they had always been slim or participated in varsity athletics (Scarmeas et al., 2002). A recent study found that professional soccer players in Italy had a 6-fold increase in ALS incidence compared to the general population (Chiò et al., 2005). However, three separate reports have reported there is no association between physical activity and ALS risk (Longstreth et al., 1998; Valenti et al., 2005; Veldink et al., 2005).

It is possible that exercise could be hazardous to ALS patients as production of reactive oxygen species, glutamatergic excitatory output, and calcium turnover at involved motor neurons all increase with physical exercise (Liebetanz et al., 2004). These potential outcomes of physical exercise are all suspect in the death of motor neurons seen in ALS. Another hypothesis involves environmental toxins and their role in the development of ALS, suggesting that physical activity resulting in continual synaptic activation may enhance transport of such toxins to motor neurons (Longstreth et al., 1991). It is also possible, considering the particular vulnerability of fast-fatigable motor units in ALS, that large amounts of intense physical exercise resulting in selective recruitment of such motor units contributes to increased incidence of disease in these athletes (Longstreth et al., 1991). However, exercise also may improve tolerance of oxygen free radicals through increases in anti-oxidants (Itoh et al., 1998). In addition,

exercise enhances new neuronal formation, decreases neurodegeneration in various diseases and ageing, and protects the nervous system through the upregulation of neurotrophic factors like IGF-1 (as reviewed by Kirkinezos et al., 2003).

Exercise studies in human ALS have been scarce, though physicians often recommend physical therapy. Rehabilitation workers report that "vigorous" physical activity appears beneficial in ALS patients by minimizing fatigue, preventing disuse weakness, and strengthening unaffected muscle (Sanjak et al., 1987). Another report showed improved strength in 14 out of 18 muscle groups following 75 days of resistance training in an ALS patient (Bohannon, 1983). A recent study found that a moderate daily exercise program resulted in less deterioration in the Ashworth spasticity scale and ALS functional rating scale (FRS), however the results were short-lived and strength parameters were not improved (Drory et al., 2001). Exercise to the level of anaerobic threshold was found to be beneficial in ALS patients currently using non-invasive methods of assisted ventilation (Pinto et al., 1999). The treatment group showed better Functional Independent Mobility (FIM) test scores, respiratory function test scores, and slower clinical progression.

The effects of exercise on mouse models of fALS have been studied more extensively, but results have been equivocal. In these studies there appears to be an intensity-dependent effect with low intensity endurance exercise producing increases in survival (Kirkinezos et al., 2003), moderate intensity exercise having no effect (Veldink et al., 2003), and high intensity endurance exercise decreasing survival in transgenic mice (Mahoney et al., 2004). Kirkinezos et al. (2003) ran mice on a treadmill at a speed of 13 meters/minute for 30 minutes, 5 days per week, and found increased survival from 129 to

139 days in male mice. They suggested the increase was due directly to neuroprotective effects of exercise on motor neurons via trophic support, or perhaps through increases in testosterone since the effect was only seen in male mice. On the other hand, the study by Veldink et al. (2003) using slightly higher intensities saw improvements in survival of female mice and not in males. Endurance exercise is known to increase levels of estrogen (Copeland et al., 2002), which has been shown to improve retrograde transport of fluorochromes in mice (Murashov et al., 2004).

To determine whether extensive exercise is harmful in ALS mice, Liebetanz et al. (2004) subjected mice to a lifetime of exercise on motor driven wheels for 10 hours daily, at distances up to 4 kilometers each day. Animals in the running group survived sedentary mice by one week, and exercise did not promote onset or progression of motor degeneration in mSOD mice. In a similar study, mice were allowed access to running wheels ad libitum for up to 12 hours each day, and mean survival was extended from 123 to 167 days (Kaspar et al., 2005). Exercise also led to improved motor function and motor neuron survival in this study. The 44-day improvement in survival is far greater than anything seen previously following exercise in this model. The discrepancy in survival duration could be explained by the fact that mice were allowed to choose a comfortable running speed and not forced into running faster than capable. In the same study, mice that ran for 2 hours daily had only a 7-day improvement in mean survival time, which was similar to what was seen in earlier exercise studies. This suggests that longer durations of low intensity exercise may be required for optimal improvements in survival.

The treatment with the greatest improvement in survival time of mSOD1 mice to date involved the combination of exercise and IGF-1 gene therapy. Kaspar et al. (2005)

allowed mice 12 hours of ad libitum exercise on running wheels each day, and injected them with AAV-IGF-1. The result was a synergistic improvement in mean survival to 209 days compared to untreated mice (119 days), exercise alone (163 days), and AAV-IGF-1 alone (141 days). These results suggest the therapeutic effects of IGF-1 in motor neurons are enhanced by exercise. It is possible that exercise allows for improved transport of the drug from the muscle to the motor neurons through improvements in neuromuscular contacts and recruitment patterns. Exercise may also help in modulation of IGF-1 binding proteins (IGFBPs). IGFBP-2, 5, and 6 are increased in the spinal cord of ALS patients, competing with IGF-1 receptors and reducing free IGF-1 (Wilczak et al., 2005). Levels of IGFBP-5, which was shown to reduce survival of motor neurons in avian embryos (D'Costa et al., 1998), were decreased following endurance training in previously sedentary men (Timmons et al., 2005).

1.3.3 Exercise, Neuroprotection, and Axonal Transport

Exercise is potentially neuroprotective because of its effects on neurotrophic factors and reduction of oxidative damage and apoptosis. Specifically, exercise has been shown to increase circulating IGF-1 levels and provide protection from brain insults in rats (Carro et al., 2001). Exercise has also been shown to increase levels of brain-derived neurotrophic factor and its receptor, TrkB (Klintsova et al., 2004), which could be important in neurotrophin protection of retrograde transport efficiency (Kuruvilla et al., 2004). Further, both neurotrophin-3 (NT-3) and BDNF mRNA levels were increased in correlation with improved axonal regeneration following peripheral axotonomy in exercised rats (Molteni et al., 2004). More importantly, BDNF and NT-3 were increased in the lumbar spinal cord following one week of treadmill exercise (Gomez-Pinilla et al.

Edgerton, 2001), suggesting that physical activity increases retrograde transport of these neurotrophins, which are also increased in skeletal muscle.

One mechanism of neurotrophic protection in motor neurons involves signalling pathways that produce anti-apoptotic signals. The exercise induced increase in IGF-1 could stimulate the PI-3K pathway leading to the downstream upregulation of Akt, a survival factor that inhibits apoptosis by inhibiting the proapoptotic substrate Bad and caspase proteases responsible for cell death (Lawlor and Alessi, 2001). BDNF promotes neuronal survival through the same pathway as IGF-1 (Yamagishi et al., 2003), and both BDNF and NT-3 are involved in axonal regeneration and growth (Gomez-Pinilla et al., 2001). While acute response to exercise involves increases in apoptosis (Phaneuf and Leeuwenburgh, 2001), a recent study found that an 8-week treadmill-running program resulted in decreased pro-apoptotic gene expression and increased anti-apoptotic gene expression in rat skeletal muscle (Siu et al., 2004). Specifically, endurance exercise resulted in increased HSP70, the antioxidant enzyme Mn-SOD, and survival factor Bcl-2 along with decreases in apoptotic markers Bax, Apaf-1, and DNA fragmentation.

Even more important for the current study is how exercise affects axonal transport, due to our current knowledge of ALS disease pathology and the exciting new treatment possibilities related to retrograde transport. Endurance exercise training in rats can cause physiological changes in motor neurons including increased oxidative enzyme levels, changes in motor units to include more muscle fibers, as well as improvements in the rate of transport of proteins via fast axonal transport (Beaumont and Gardiner, 2003). Regular endurance training also resulted in increased calcitonin gene-related peptide (CGRP) transport from rat motor neurons to neuromuscular junctions via fast-axonal

transport (Gharakhanlou et al., 1999). CGRP may be instrumental in the remodelling that takes place at the neuromuscular junction following exercise, specifically with increased synthesis of acetylcholine receptors (AChR) and the globular (G4) form of acetylcholinesterase (AChE). Finally, endurance exercise training increased fast-anterograde axonal transport by 30%, and showed particular increases in the transport of SNAP-25 to the synapse (Kang et al., 1995). SNAP-25 has an apparent role in axonal elongation, and therefore could facilitate the sprouting response of motor neurons following denervation.

In attempting to treat ALS, it makes sense to exploit the remarkable plasticity of the neuromuscular system. Following motor neuron death or partial axotomy, surviving motor neurons produce "sprouts" to reinnervate neighbouring denervated muscle fibers (Gordon et al., 2004). This adaptive axonal sprouting results in up to three- to five fold increases in motor unit size as motor neurons sprout to reinnervate denervated fibers (Tam and Gordon, 2003a). These enlarged motor units can compensate for the loss of up to 85% of functional motor neurons (Gordon et al., 2004). A controversial issue is whether exercise promotes or inhibits the sprouting response. Some research indicates that the sprouting response to denervation is improved by increased daily activity (Einsiedel and Luff, 1994; Gardiner et al., 1984; Seburn and Gardiner, 1996). Following partial denervation, exercise increased motor unit force production by 28% without increasing cross-sectional area of muscle fibers, leading researchers to believe sprouting had occurred to increase the size of the remaining motor units (Seburn and Gardiner, 1996).

Recent evidence, however, suggests that neuromuscular activity could be detrimental to the sprouting response following axotomy (Love et al., 2003; Tam et al., 2001; Tam et al., 2002a; Tam and Gordon, 2003b). In extensively denervated rat muscles, motor unit enlargement and sprouting were significantly reduced following 1 month of 1751 meters/day voluntary wheel running (Tam et al., 2001). It was further shown that the neuromuscular activity prevented the bridge formation between Schwann cell processes at innervated and denervated endplates (Tam and Gordon, 2003b). One proposed mechanism for this effect is a down-regulation of glial fibrillary acidic protein (GFAP), an important stimulator of perisynaptic Schwann cell processes, by increases in acetylcholine and calcium levels at the nerve terminals during exercise. Similarly, electrical stimulation of muscle following denervation inhibited the ability of terminal Schwann cells to form or maintain bridges with innervated synaptic sites, and prevented growth of axons along processes extended by Schwann cells (Love et al., 2003). However, this is likely a result of direct stimulation of the muscle because with this method denervated muscle fibers are being activated, thereby counteracting the sprouting stimulus. In an early study where denervated muscle was stimulated through the nerve, terminal sprouting persisted (Brown and Holland, 1979).

Inactivity following denervation also appears to be detrimental to the sprouting response. Axonal sprouting is induced in normally innervated muscles by muscle inactivity through tetrodotoxin (TTX) induced pre-synaptic and α-bungarotoxin (α-BTX) induced post-synaptic blockade of neuromuscular activity (Tam and Gordon, 2003a). However, following partial denervation by axotomy, TTX- (Tam et al., 2002b) and α-BTX- (Connold and Vrbova, 1991) induced inactivity prevents motor unit enlargement

through severe reductions in the sprouting response. Therefore, at least some level of activity is necessary for proper sprouting to occur following denervation. It is important to point out that the level of denervation affects the activity-induced reduction in sprouting. In recent studies where detrimental effects of activity on sprouting responses were found (Brown and Holland, 1979; Love et al., 2003; Tam and Gordon, 2003b), only extensively denervated muscles (>80% partial denervation) were negatively affected whereas the same effect was not seen in moderately denervated muscles (~50% partial denervation) (Tam and Gordon, 2003a). The studies where exercise had positive effects on axonal sprouting (Einsiedel and Luff, 1994; Gardiner et al., 1984) were looking at moderately denervated muscles, and the effects were mostly seen in fast twitch muscles. With ALS being a disease where progressive denervation takes place and fast twitch fibres are particularly affected, exercise could have beneficial effects on sprouting, thereby increasing the time before extensive and irreversible damage (>80% denervation) takes place.

CHAPTER 2: EFFECT OF EXERCISE ON RETROGRADE TRANSPORT RATE IN A MOUSE MODEL OF ALS

2.1 Justification of Study

ALS is a disease of complex and yet unknown causes. A variety of potential pathological events have been indicated in the research, including amino acid excitotoxicity, neurofilament aggregation, free radical damage, apoptotic cell death, and peripheral distal axonopathy among others. A few of the current proposed treatments involve the retrograde delivery of neurotrophic factors from the muscle to the dying motor neurons. A potential problem for successful human trials is that retrograde transport rates are likely decreased as ALS progresses, potentially resulting from a "dying back" axonopathy. Exercise improves neurological functioning through direct improvements in energy metabolism, and indirectly via increases in trophic substances in the circulation and the transport of those trophic substances from the muscle. Exercise has been shown to extend life in a transgenic mouse model of ALS through unknown mechanisms. It is possible that exercise could improve motor neuron survival in ALS via improvements in the retrograde transport of neurotrophic factors from the muscle. Exercise may improve retrograde transport by improving the integrity of neuromuscular junctions. This could occur because of activity-induced sprouting leading to reinnervation or simply maintaining innervation due to repeated activation of the synapse. To my knowledge, no studies have examined the effects of exercise on retrograde transport rates or neuromuscular junction stability in ALS. The results of the following experiments

provide insight for the efficacy of exercise as a treatment for ALS and/or facilitator of retrograde transport for use in gene therapies.

2.2 Objectives and Hypotheses

Objectives:

1. To better understand the pathological mechanisms underlying amyotrophic lateral sclerosis (ALS), particularly the diminished retrograde transport rates.
2. To investigate whether exercise can improve retrograde transport rates in a mouse model of ALS, and whether those improvements are related to changes in innervation status at the neuromuscular junction.
3. To determine whether exercise will improve motor function in ALS mice, and whether those improvements are correlated with improvements in retrograde transport.

Research Hypotheses:

Research Hypothesis #1

Endurance exercise training of a mouse model of ALS will attenuate the diminished retrograde transport rates seen in affected animals relative to their wild-type controls.

Research Hypothesis #2

The alterations in retrograde transport rates of fluorochromes in a mouse model of ALS due to exercise will be related to reduction in denervation at the neuromuscular junction.

2.3 Methods

2.3.1 Animals

Transgenic mice expressing the mutated SOD1 gene [B6SJL-TgN(SOD1-G93A)1Gur] originally obtained from Jackson Laboratory (Bar Harbor, ME) were locally bred from such progenitor stock at the Animal Care Facility (Simon Fraser University). These mice model human fALS by overexpressing human Cu/Zn-SOD1 gene with a missense mutation G93A. This same mutation has been found in cases of human fALS (Gurney et al., 1994). These mice exhibit symptoms that are clinically and pathologically similar to ALS in humans. Symptoms usually begin between 80 and 90 days of age with hindlimb tremor in early stage and progress to partial and full paralysis of hindlimbs near endstage, which typically occurs around 130 days. An artificial end stage is used for ethical reasons and occurs when the mouse can no longer right itself from lateral recumbence within 10 seconds.

The mouse colony is maintained by breeding SOD1 affected males with unaffected (wild-type) females. Litters were weaned and genotyped via PCR of ear punch sample at 28 days of age. Mice were monitored daily for disease symptoms and classified as asymptomatic, mild, moderate, or severe based on a set of disease characteristics (see Appendix A). Mice were kept under a 12 hour light/dark cycle with dry food pellets and water available *ad libitum*. A total of 35 mice were sacrificed; n= 8-10 per group in 4 groups based on disease and exercise status. G93Ased and WTsed groups were restricted to normal cage activity while G93Aex and WTex groups underwent the exercise treatment described below.

2.3.2 Exercise Training

Transgenic mice and wild-type littermates were randomized into exercise and sedentary groups (n= 8-10). Exercise groups began running on a motor driven treadmill at 56 days of age, preceded by a week of familiarization starting at 50 days of age, and continuing for 8 weeks (Figure 1). Treadmill running began at a speed of 10 m/min for 10-15 minute sessions and progressed to 40 minutes of continuous running at 13 m/min, 5 days per week, similar to previous exercise studies in this mouse model (Kirkinezos et al., 2003; Veldink et al., 2003). At this speed mice run at a mean of 78% of their maximal oxygen consumption (VO2) (where VO2 = 0.127 x weight + 0.040 x running speed – 0.974) (Fernando et al., 1993; Schefer and Talan, 1996).

When some of the ALS-affected mice could no longer maintain a speed of 13 m/min, the speed was slowed to a pace they could sustain and duration was increased to obtain 500 ± 30 meters in total distance run per day, but in all cases total exercise time did not exceed 1 hour per day. If mice could no longer run at a minimum of 7m/min, they remained sedentary until sacrifice. However, almost all mice were able to complete the exercise training protocol. The exercise treadmill is equipped with a motivation grid containing compressed air and weak electrical current. Mice were stimulated to run via puffs of air and/or small electrical shocks. Running performance was monitored based on number of failures during a 10-minute exercise period. Exercise took place during the 12-hour dark cycle when mice are typically most active, usually about 3 hours into the cycle since mice typically feed at the beginning of that cycle. In an effort not to disturb their daily cycle, the mice were exercised in a dark room with only a red light used for monitoring purposes. See Figure 2 for pictures of treadmill setup.

2.3.3 Determination of Animal Genotype

2.3.3.1 Polymerase Chain Reaction (PCR)

At approximately 28 days of age, small ear tissue punches were taken from each mouse and placed in an autoclaved 1.5 mL eppendorf tube with 150 µl of Chelex solution to prepare the DNA template for the polymerase chain reaction (PCR) protocol. The Chelex solution consisted of Chelex (5% w/v; Fluka, product #95577), Proteinase K (2µg/µl; Sigma Aldrich, product #P-2308), and RNase A (10µg/µl; Sigma Aldrich, product #R-4876). Tubes were placed in a 55°C water bath for 15 minutes, vortexed for 10 seconds, then placed back in the water bath for another 15 minutes. Tubes were vortexed for 10 seconds and then placed in a dry heating block set at 100°C for 8 minutes. Samples were vortexed for 10 seconds and then centrifuged for 15 minutes at 12000 rpm (Baxter Canlab Biofuge A). The supernatant from each sample was then transferred into a new, autoclaved eppendorf tube with autoclaved pipette tips. The supernatant was then either used immediately as a template for the PCR protocol or stored at 20°C.

PCR protocols previously described (Hu et al., 2003) were used to differentiate genotypically "affected" mice from wildtype mice by whether or not the G93A transgene was expressed. Small volumes of PCR mixture were prepared (1.0µl DNA template, 0.5µl Primer mSOD1, 0.5µl Primer mSOD2, 0.5µl $MgCl_2$, 2.5µl of 10X buffer, 2.5µl of dNTPs, 0.3µl Taq Polymerase, and 17.2µl of distilled water; Qiagen product #201203). The primers were: mSOD1- CAT CAG CCC TAA TCC ATC TGA (forward) and mSOD2- CGC GAC TAA CAA TCA AAG TGA (reverse), both obtained from Invitrogen. To amplify the DNA, PCR was conducted on the Gene Amp PCR system

2400 (Applied Biosystems) using the following settings: 1 cycle for 5 minutes at 95°C; 30 cycles for 30 seconds each at 94°C, 56°C, and 72°C; 1 cycle for 30 seconds at 94°C and 56°C; then 10 minutes at 72°C, the solution is then cooled down to 4°C and kept there indefinitely.

2.3.3.2 Gel Electrophoresis

The PCR product was prepared for DNA electrophoresis by combining 1µl of loading dye with 10µl of sample. The mixture was then loaded into a 1% agarose gel (containing 0.01% Ethidium Bromide) along with positive and negative controls. Gels were run for 30-45 minutes at 100V in a Mupid-21 Mini Gel Migration Trough (Cosmo Bio Co. Ltd) containing 0.5X Tris-Acetate-EDTA (TAE) buffer. Gels were then placed under U/V light to determine the presence or absence of the mutant human SOD1 transgene (236 base pairs). If there was any questionable samples (i.e. fragmented or light bands), they were re-run to confirm genotype.

2.3.4 Neuromuscular Function Tests

To look for functional improvements that may accompany exercise training, two common neuromuscular tests were performed once per week starting at 8 weeks of age: the rotorod test and the modified paw grip strength endurance (PaGE) test.

2.3.4.1 Rotorod Test

For rotorod testing mice were placed on the rotorod rotating at a constant speed of 20 rpm, and evaluated based on time they can stay on the rod without falling off, with a (maximum of 3 minutes) (Turner, 2004). Time was stopped if the mouse held on to the

rod without walking for two consecutive revolutions. The average of two trials was taken as the score for each week. Testing occurred on days the mice did not perform exercise training.

2.3.4.2 Modified Paw Grip Strength Endurance (PaGE) Test

The PaGE test measured the amount of time a mouse could hang upside down from a cage lid or similar wire mesh. It involves all four limbs and has been shown to correlate with motor deficits in mSOD mice (Weydt et al., 2003). The test starts with mice on top of the cage lid, which was then shaken causing the mouse to hold on to the bars. The apparatus was quickly flipped over and the timer started. The time it takes for the mouse to fatigue and drop down from the bars was measured, with the maximum being 90 seconds. The average of two trials was recorded as the score for each week. Testing occurred on days mice did not perform exercise training.

2.3.5 Injection of Fluorescent Dye

All analyses were performed 3 days following the last training session in order to control for acute exercise effects. For the injection of the retrograde tracer, 115-day old mice were anaesthetized in an induction chamber via a 5% Isofluorane flow. Once induced, mice were maintained at a rate of 2-2.5% for the duration of the procedure. Exercise and sedentary affected G93A mSOD mice and their age-matched exercise and sedentary controls were all injected with 5μL of 2% Fluoro-Gold solution (in sterile saline; Fluorochrome Inc.) through a 1mm incision above the belly of the shaved right medial gastrocnemius muscle using a 33-gauge needle with a 10μL gastight Hamilton

syringe (#1702) whose position was controlled with a micromanipulator (see Figure 3). The 5μL injection occurred over a 2-minute period, and the needle was left in the muscle for 1 minute and withdrawn slowly to prevent withdrawal of any of the tracer via capillary action. Polysporin was then applied to the incision and mice put on 100% oxygen until they awoke. They then recovered under a heated lamp for 30 minutes before being placed in their cage.

2.3.6 Euthanasia and Tissue Harvesting

Animals survived for 24 hours after the injections at which point they were euthanized with a CO_2/O_2 mixture. About 45 seconds after they stopped breathing but before cardiac arrest, they were perfused transcardially with 30 mL of 1X phosphate buffered saline (PBS, pH 7.4) followed by 30 mL of 4% paraformaldehyde (pH 7.4). The spinal cord and right and left gastrocnemius muscles were then carefully dissected and further fixed in 4% paraformaldehyde before being cryoprotected in 20% sucrose (w/v in 1X Phosphate buffered saline) for 24 hours.

2.3.7 Immunohistochemical Analysis: Spinal Cord

2.3.7.1 Cryosectioning

The lumbar spinal cord was mounted with optimal cutting temperature (OCT) medium (Tissue Tek) and sectioned transversely on a Reichert-Jung Cryocut 1800 (Leica) using Feather microtome blades (Tissue Tek) at 30 μm and placed in 1X PBS for further processing. The cryostat temperature was set at – 15-17°C.

2.3.7.2 Immunofluorescent Staining

Free-floating 30 μm sections were counterstained with antibodies to choline acetyltransferase (ChAT) to identify motor neurons in the sciatic pool of both sides of the spinal cord (for full protocol see Appendix C). Sections were rinsed in 1X PBS, then permeabilized with 0.4% Triton X. Sections were then incubated in a blocking solution containing 2% normal serum and 5% bovine serum albumin (BSA) at room temperature for 1 hour. The sections were then incubated in the primary polyclonal antibody (1:200, goat anti-ChAT, Chemicon International) for 48 hours at 4°C. Sections were then incubated for 2 hours in the secondary antibody at room temperature (1:200, donkey anti-goat Alexa Fluor 555).

After several washes in 1X PBS, sections were mounted on glass slides (Fischer Scientific) and air-dried in the dark. Once dry, Vectashield Mounting Medium for Fluorescence (Vector Laboratories Inc.) was added to the slide before applying cover-slips. Cover-slips were sealed with clear nail enamel and slides were stored in a dark box at 4°C. Some slides were made containing sections that only had been labelled with secondary antibody to serve as controls when determining auto-fluorescence during the imaging process.

2.3.7.3 Imaging

Sections were visualized using an Olympus BHX40 wide-field epifluorescent microscope. A U-MWU2 wide-band UV filter (excitation filter: 330-385 nm, emission filter: 420nm; Olympus) was used for Fluoro-Gold imaging (excitation: 360 nm, emission

peak: 620 nm) and a U-MWIG wide-band cube filter (excitation filter: 530-550nm, emission filter: 570 nm, Olympus) was used for ChAT/ Alexa Fluor 555 imaging (excitation 555nm, emission peak: 565 nm). Images were acquired using a CoolSnap CCD camera (RS Photometrics) and processed with MetaVue 4.6 software (Universal Imaging Corp). Images were collected at 20X and 40X, with images collected at 40X used for counting of ChAT-positive and Fluoro-Gold-positive cells. Background fluorescence levels were determined for ChAT using several sections exposed only to secondary antibodies and averaging the intensity levels. It was found that the blocking procedure used was sufficient to preclude the need to remove of background fluorescence.

2.3.7.4 Image Analysis

Motor neurons of the sciatic pool were identified via the average number of ChAT positive neurons counted in laminae IX in the 2mm long L3 to L6 region of the lumbar spinal cord in the side ipsilateral to Fluoro-Gold injections. Sections were cut at 30μm and every fourth section was analyzed to avoid any double-counting of motor neurons whose cell bodies can reach up to 100μm in length. Eight sections were chosen at random from the remaining sections for counting. Sections were counted blind to genotype and exercise training. The percentage was then calculated for each section and averaged across the eight counted to yield a final percentage of motor neurons displaying Fluoro-Gold for each animal. Retrograde transport rates were defined by the percentage of ChAT positive motoneurons also showing Fluoro-Gold staining. This indicated efficient retrograde transport in those motor neurons, though it may be more appropriate

to refer to this as a labelling index (Wagey et al., 2001). Representative images of Fluoro-Gold positive cells, ChAT positive cells, and the overlay are demonstrated in Figure 4.

2.3.8 Immunohistochemical Analysis: Neuromuscular Junctions

2.3.8.1 Cryosectioning

Gastrocnemius muscles were removed from storage and treated with 0.5M glycine solution for 1 hour to quench the paraformaldehyde fixed tissue, eliminating potential antigen cross-linking. Whole muscles were individually mounted in optimal cutting temperature (OCT) medium (Tissue Tek) and 40μm thick sections were cut longitudinally using a Leica CM1900 UV Cryostat at a temperature of –16-20°C. Sections were immediately transferred to poly-L-lysine (Sigma) coated glass slides (Fischer Scientific) and immersed in ice-cold acetone for 10 minutes for tissue fixation.

2.3.8.2 Immunofluorescent Staining

Slides were placed in a humidifier and tissue sections were surrounded with a liquid blocker (Super PAP pen, Pelco). Sections were then washed with 1X PBS and non-specific binding was blocked for 1 hour at room temperature with blocking buffer (3% Normal Goat Serum; 5% BSA; 1% Fab Fragment Goat Anti-Mouse IgG; 0.1% TritonX; completed to desired volume with 1X PBS). Sections were double-labelled with primary monoclonal antibodies to neurofilament (NF160, 1:200, Chemicon) and synaptic vesicle protein (SV2,1:50, Iowa Developmental Hybridoma Bank) at 4°C for 24 hours. Sections were then washed 5x5 minutes in 1X PBS before being incubated in secondary antibodies, cy3-labeled goat anti-mouse (1:200, Jackson ImmunoResearch)—SV2 and NF and fluorescein-α-bungarotoxin (α-BTX, 1:200, Biotium)—NMJ, for 90 minutes in

the dark at room temperature. Sections were then washed 5x5 minutes with 1X PBS and air-dried. Once dry, Vectashield mounting medium (Vector Laboratories) was added to each section of each slide before applying cover-slips and sealing with clear nail enamel. Slides were then stored at 4°C in a dark box until imaged.

2.3.8.3 Imaging

Muscle sections were imaged with a Leica DM 4000B epifluorescent microscope. A FITC Brightline Filter set (Leica #3540B) was used when imaging FITC-labelled cells (emission peak at 530nm), and a Texas Red Brightline Filter set (Leica #4040B) was used for imaging Cy-3-labelled cells (emission peak at 620nm). Images were acquired with a Leica DFC 350FX camera and Leica Application Suite software at both 20X and 40X magnifications. All sections with similar labelling (NF/SV2 or α-BTX) were imaged under constant conditions (exposure time, intensity, and contrast) in order to limit variability for comparison purposes. Background fluorescence was eliminated using average intensity obtained from sections stained with secondary antibody alone.

2.3.8.4 Image Analysis

As per previous studies (Fischer et al., 2004; Schaefer et al., 2005) neuromuscular junctions were identified by labelling muscles with α-bungarotoxin which binds specifically to post-synaptic acetylcholine receptors at the motor end plates. Monoclonal antibodies to neurofilament (NF160) and synaptic vesicle protein (SV2) were used collectively to identify axons and terminal branches that innervate the neuromuscular junctions.

Approximately 200 neuromuscular junctions were counted and assessed per animal (n=5 per group, G93Aex, G93Ased, CONex, CONsed). In order to count a representative sample of sections from throughout the muscle, every 10^{th} section was analyzed and a total of 5 muscle sections were counted per animal. For consistency, only the medial portion of each gastrocnemius was analyzed, enabling a more representative sample of the entire muscle. For each neuromuscular junction identified, a classification was made based on a visual interpretation of its innervation status (Figure 5). NMJs (green) were scored as "innervated" if there was complete overlap with an axon terminal (red), "denervated" if the NMJ was not associated with a nearby axon, or "intermediate" if there was partial overlap with an axon terminal or only a pre-terminal axon nearby.

To control for evaluator bias, a second evaluator was trained in the classification system and asked to assess the same sections as the primary investigator. Both the primary and secondary investigators were blinded to the treatment status of the mice (exercise vs. sedentary) from which samples were being analyzed. Additionally, the secondary investigator was blinded to the condition (G93A vs. WT). Inter-rater reliability was assessed and determined to be highly correlated in regards to the percentage of muscle fibers scored as innervated (r=0.99), denervated (r=0.94), and intermediate (r=0.79).

2.3.9 Data Analysis

2.3.9.1 Animals

G93A mice were age-matched to wild-type controls and randomly assigned to exercise or sedentary groups. All mice were euthanized at 115 days of age, regardless of

symptomotology. Body mass, rotorod and PaGE test performance, and symptoms were assessed weekly. Independent t-tests (one-tailed, α-level = 0.05) were used to compare means between each G93A group (ex vs. sed) and their age-matched wild-type controls. The mean age of symptom onset was calculated based on the age when animals first showed clinical signs based on the criteria set by the staff at the Animal Care Facility (Appendix A). Analysis of covariance (ANCOVA) was used to examine the relationship between age of symptom onset and stage of disease progression reached at 115 days of age in the exercise and sedentary groups. To test for differences in performance on rotorod and PaGE tests between groups, chi-square and Fischer's exact tests were used.

2.3.9.2 Immunohistochemistry

Independent t-tests were used to determine the differences between groups for both percentage of motor neurons displaying Fluoro-Gold and percent innervated, intermediate, and denervated muscle fibers. A two-way 2 (disease) by 2 (training) randomized groups ANOVA was conducted to compare the group means of the percentage of motor neurons displaying Fluoro-Gold. A Pearson correlation (r) statistic was performed to examine the relationship between innervation status at the neuromuscular junction and retrograde transport of Fluoro-Gold to motor neurons. All analyses were performed using SPSS 15.0 for Windows.

2.4 Figures

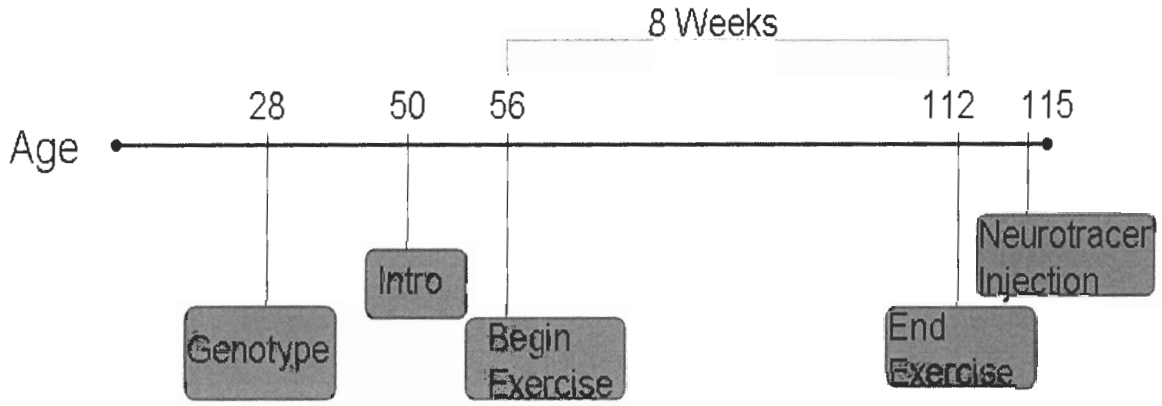

Figure 1 Study design timeline

Transgenic G93A mice were identified via genotyping of ear-punch sample at 28 days of age. Mice were randomly assigned to exercise and sedentary groups. All groups were introduced to the treadmill at 50 days of age. The treatment groups began exercise at 56 days of age, continuing for 8 weeks. Three days following the final exercise session, all mice were injected with the neurotracer Fluoro-Gold to measure retrograde transport. Mice were euthanized 24-hours post-injection and tissue was harvested for analysis.

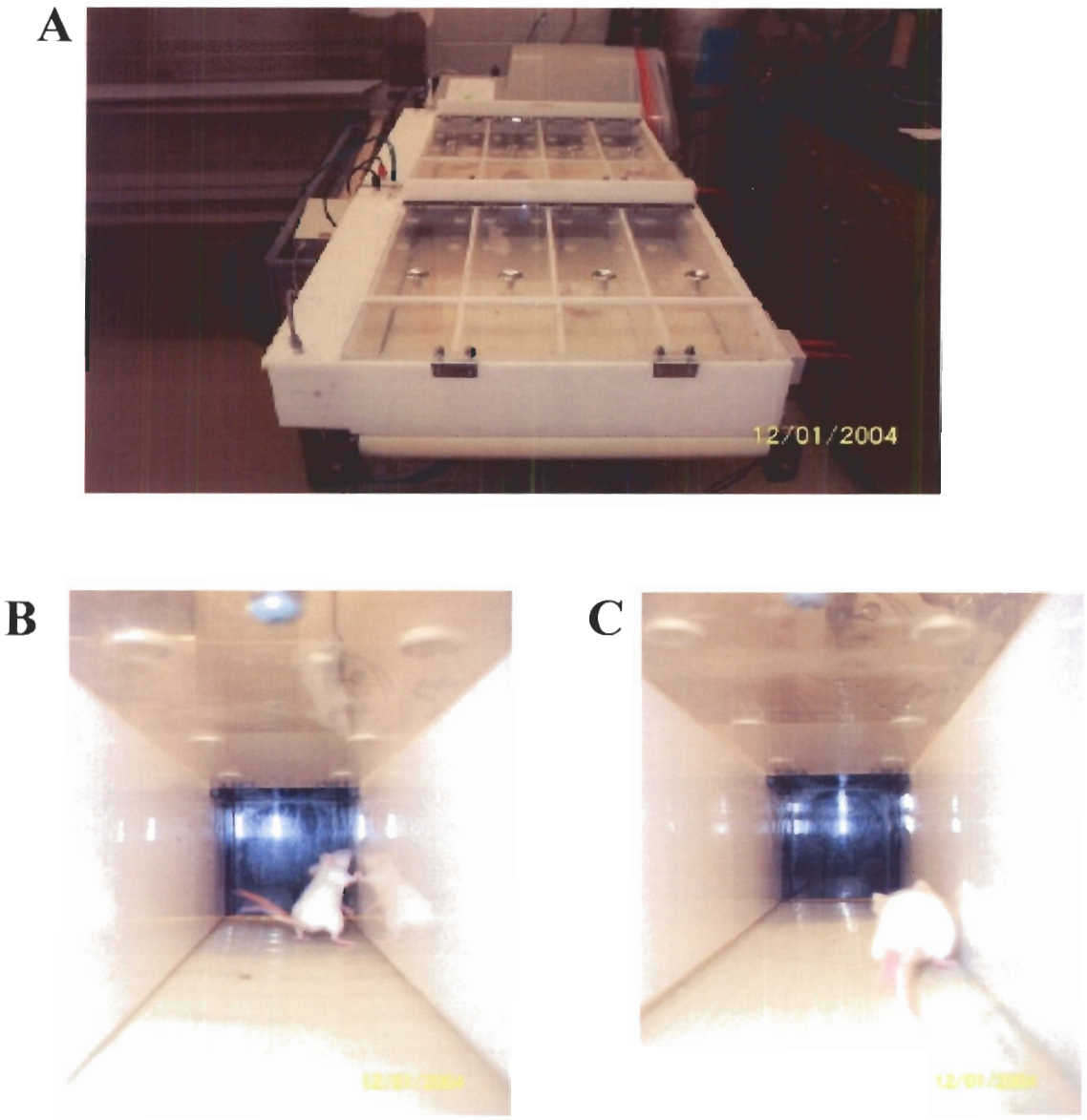

Figure 2 Treadmill setup and WT and G93A mice running on treadmill.

(A) Eight mice were run at a time in lanes. At the back of each lane there is a motivation grid with puffs of air and small electrical shocks. A WT mouse is shown running without problem (B), while an affected mouse is shown with a hunched back, posture very low to the ground, and a paddling gait (C).

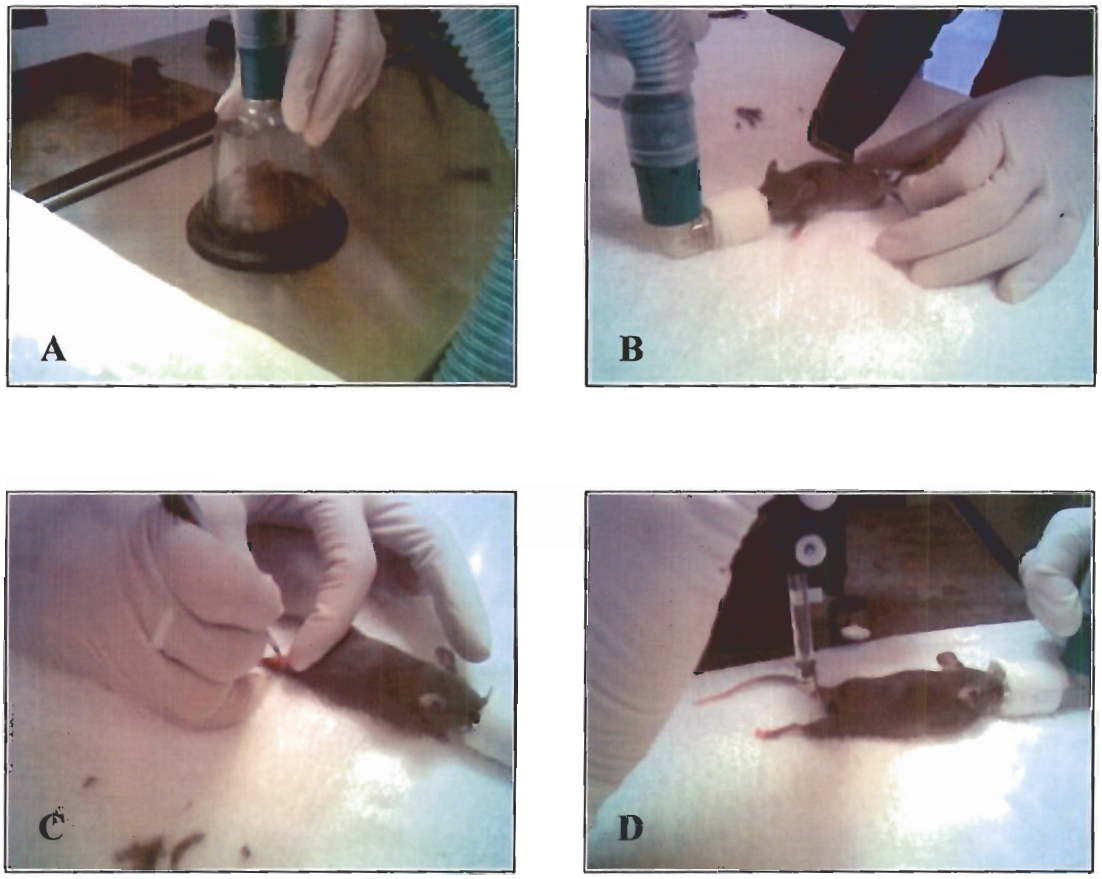

Figure 3 Fluoro-Gold injection procedure[1].

Animals were anesthetized in an induction chamber with 5% Isoflurane. Animals were then placed on a respirator with a 2.5% isoflurane flow rate. The right calf area was shaved and a 1mm incision above the belly of the gastrocnemius was made with a scalpel. 5µl of 4% Fluoro-Gold was then injected directly into the superficial medial gastrocnemius over a 3 minute time period with a Hamilton syringe controlled by a micromanipulator. After the injection the 26-gauge needle was left in the muscle for 1 minute to prevent the uptake of any Fluoro-Gold via capillary action. Polysporin was applied to the incision and the animals recovered under heating lamps for 30 minutes before being returned to their cages.

[1] Adapted with permission from the thesis of I.J.L. McFee (M.Sc. 2005, SFU)

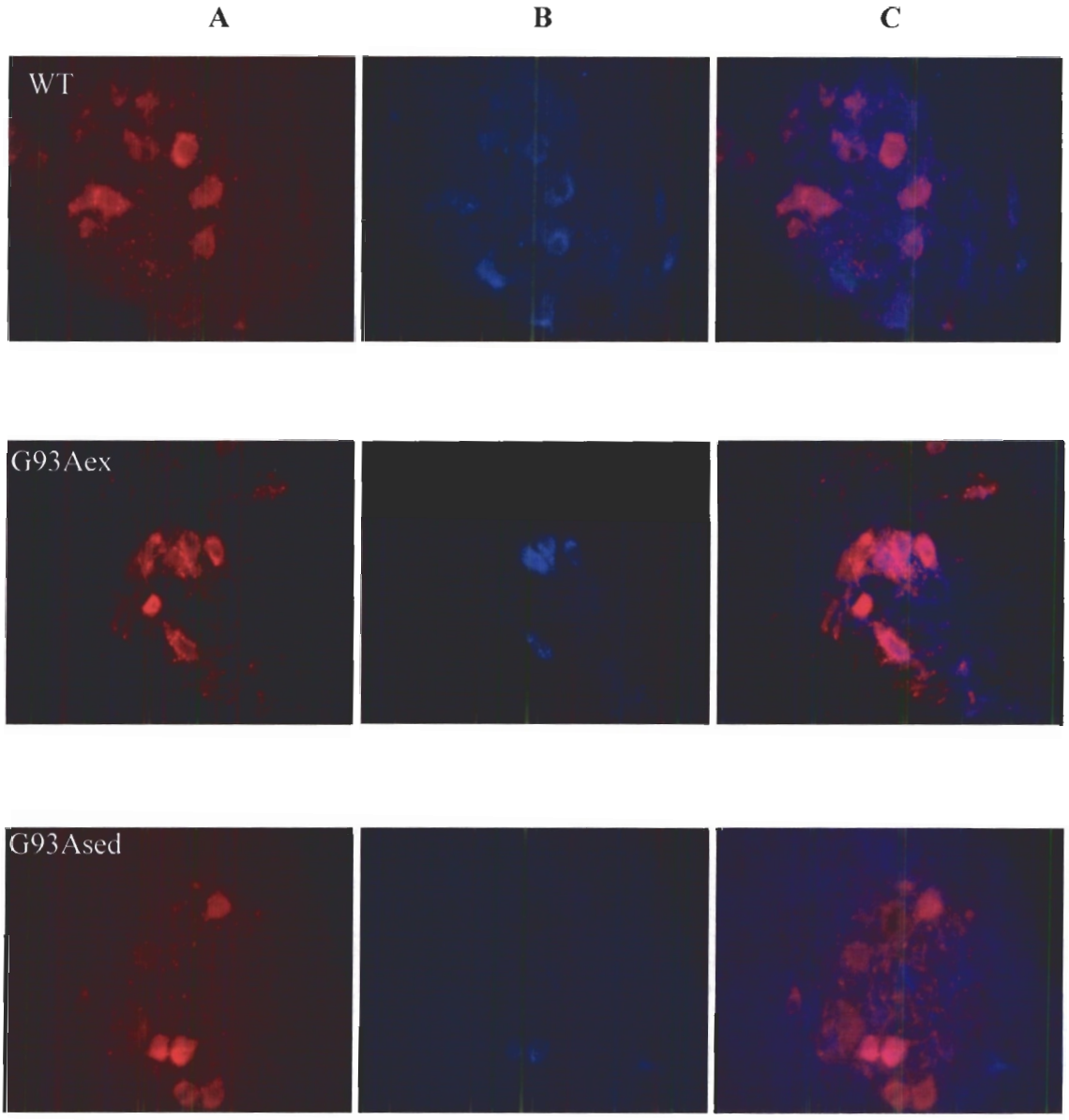

Figure 4 Spinal cord sections labelled for ChAT and FG.

The anterior horn region of lumbar spinal cords from WT mice and affected G93A mice are shown labelled with choline acetyl-transferase to identify motor neurons (A), Fluoro-Gold to represent relative retrograde transport in 24-hour period (B), and the overlay of the two images to show the ratio of ChAT positive motor neurons also positive for FG (C).

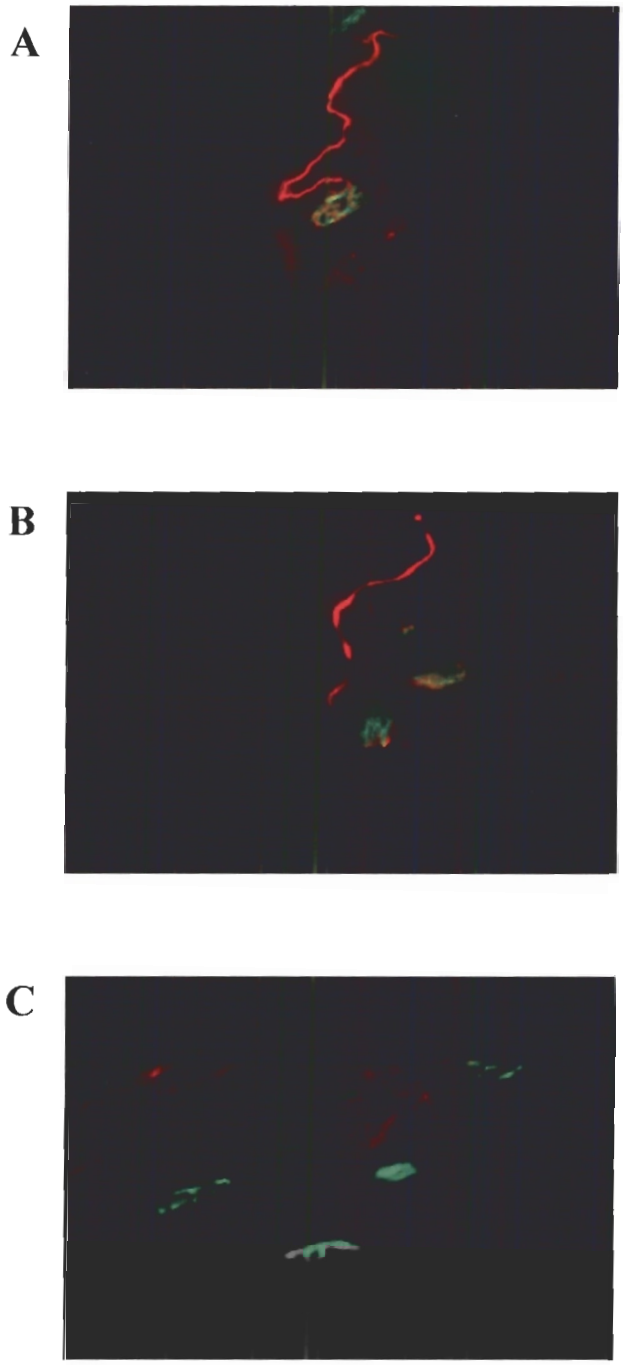

Figure 5 Immunohistochemistry of neuromuscular junctions.

The medial gastrocnemius muscles were sliced and sections were stained with antibodies to neurofilament and synaptic vesicle protein (red) to show axons, and α-bungarotoxin to identify neuromuscular junctions. Junctions were scored as Innervated (A), Intermediate (B), or Denervated (C).

2.5 Results

2.5.1 Animals

As shown in Table 1, mean body mass was significantly different between WT and G93A mice at death in both males and females ($p<0.01$). This difference was not significant in male mice at 12 weeks of age ($p=0.07$), but was significant by 13 weeks ($p<0.05$) corresponding with symptom onset. Female WT mice weighed significantly more than age-matched female G93A mice beginning at 13 weeks of age as well ($p<0.05$). Figure 6 shows the mean body mass for all mice.

The mean body mass at death was significantly higher in sedentary G93A mice (G93Ased) compared to those who underwent exercise training (G93Aex) ($p<0.01$). This difference was due to the male mice ($p<0.05$) as there was no significant difference in the female groups ($p= 0.25$). Although sedentary mice weighed more, they lost more weight between 13 weeks of age and death than mice who exercised (average loss of 1.51 ± 1.1g vs. 0.43 ± 1.0g; $p=0.07$). This difference was only seen in male mice.

The average age of symptom onset for affected mice was 81 ± 11 days, though there was a considerable range (65-101 days). Age of onset was not significantly different between sedentary mice (80 ± 9 days) and those who underwent the exercise program (81 ± 11 days). Mice were categorized according to stage of disease progression when euthanized (115 days) with the use of a scale of 1-4 based on observed symptoms (1-moderate, 2-late moderate, 3-advanced, 4-endstage). These stages are described in Appendix A. There was no effect of exercise on the average stage of disease progression at 115 days. However, a Spearman's correlation was performed, showing a strong relationship between age of onset and the stage mice progressed to before sacrifice at 115

days (r = -0.80) in sedentary mice. In mice who underwent exercise training there was no significant relationship between age of onset and disease progression (r = -0.32). This result suggests that exercise may delay disease progression in some mice. However, analysis of covariance (ANCOVA) revealed there was no significant difference between the slopes of the regression lines (F=0.67, p=0.43), indicating no significant effect of exercise on disease progression in G93A mice. Figure 7 shows a scatter plot comparing the stage of disease progression before sacrifice at 115 days with the age of onset of disease symptoms for individual G93A affected mice.

2.5.2 Neuromuscular Function

2.5.2.1 Rotorod Test

Rotorod performance was evaluated once per week with the average of two trials being reported as the score with a maximum of 180 seconds. Mice were considered to complete the rotorod protocol for the week if the average for the two trials was greater than 160 seconds. The percentages of mice completing the rotorod protocol from weeks 12-16 are shown in Figure 8. At weeks 15 and 16, only 30% of G93Ased mice could complete the rotorod where 50% of G93Aex mice could. However, chi square and Fisher's exact tests showed these percentages were not significantly different (p=0.65). Both G93A groups performed significantly worse on average than WT mice at 16 weeks ($p<0.001$) and beginning at 13 weeks of age ($p<0.05$) as assessed using Student's T-tests.

2.5.2.2 Paw Grip Strength Endurance (PaGE) Test

G93Aex mice did not show significant improvement in mean PaGE test performance at 16 weeks of age over G93Ased mice (p=0.18). PaGE test performance was evaluated once per week. The average of two trials was taken with a maximum of 90

seconds. Mice able to hang onto the cage for an average of more than 80 seconds were considered to have completed the protocol for that week. Figure 9 shows the percentages of mice completing the PaGE protocol from weeks 12-16. At week 15, 40% of G93Ased and 67% of G93Aex mice were able to complete the protocol, and at week 16, 30% and 50% respectively were able to complete the protocol. Chi square and Fisher's exact tests determined these percentages were not significantly different ($p=0.65$). G93Ased mice performed significantly worse on average than WT mice beginning at 14 weeks (76.5 ± 15.6s vs. 90.0 ± 0s; $p<0.05$), whereas the performance of G93Aex mice was still not significantly different from WT at 15 weeks (70.7 ± 22.8s vs. 90 ± 0s; $p=0.057$). Both G93A groups performed significantly worse than WT mice at 16 weeks (57.0 ± 29.7s and 40.0 ± 36.1s vs. 90.0 ± 0s (WT); $p<0.05$).

2.5.3 Immunohistochemistry

2.5.3.1 Retrograde Transport of Fluoro-Gold

As shown in Figure 10, retrograde transport rate, as measured by mean percentage of motor neurons displaying Fluoro-Gold 24-hours after intra-muscular injection, was significantly higher in G93Aex mice than G93Ased mice ($p<0.01$). Wild-type mice who underwent exercise training (WTex) did not show a significant increase in mean percentage of motor neurons displaying Fluoro-Gold compared to sedentary wild-type mice (WTsed) ($p=0.06$). Both groups of G93A mice showed significantly less motor neurons positive for Fluoro-Gold than age-matched WT mice ($p<0.001$). There was a significant interaction effect between treatment status and disease status ($F=4.16$, $p=0.05$) indicating that absence of disease reduces the effect of exercise on improvement in retrograde transport.

There was a significant gender affect in G93A mice with retrograde transport. Females who underwent exercise training were not significantly higher than sedentary females (55.3 ± 6.2% vs. 49.0 ± 2.7%; p=0.07), whereas retrograde transport significantly improved in male G93A mice who underwent exercise training (56.6 ± 1.8% vs. 46.6 ± 5.7%; p<0.01). However, overall there was no significant difference between male and female G93A mice (52.8 ± 5.9% vs. 50.0 ± 6.8%, respectively; p=0.39).

Data are also represented in Table 2 as total number of ChAT- and FG-positive motor neurons counted per animal. For each animal, eight sections selected at random were analysed and the mean total motor neurons counted per animal are shown. It was found that both G93Ased and G93Aex had significantly fewer ChAT-positive and FG-positive motor neurons than wild-type mice (p<0.01). G93Ased mice had significantly fewer FG-positive motor neurons than G93Aex mice (p<0.05).

2.5.3.2 Innervation Status at the Neuromuscular Junction

Figure 11 shows the effect of exercise on innervation status at the neuromuscular junction. G93Aex mice had a significantly higher percentage of intact neuromuscular junctions (those identified as innervated) compared to G93Ased mice (p<0.01). Both groups had a similar amount of neuromuscular junctions scored as intermediate (p=0.54), while G93Aex mice had a significantly lower percentage of denervated junctions (p<0.01). Both groups displayed significantly less innervation than WT mice (p<0.001). See Table 3 for mean number of innervated, intermediate, and denervated fibers counted per group.

The differences seen in relative retrograde transport rates between G93Aex and G93Ased mice could be related to the level of innervation still intact at the

neuromuscular junction. There exists a strong positive relationship between retrograde transport rate and percent innervation ($r = 0.75$, $p<0.01$) (Figure 12), and a negative relationship between retrograde transport rate and percent denervation ($r = -0.64$, $p<0.05$).

2.5.4 Tables and Figures

Table 1 **Mean body mass from weeks 12-16 in WT, G93Ased, and G93Aex mice by gender. All values reported as mean ± standard deviation.**

Group	Gender	Week 12	Week 14	Week 16
WT	Male	25.9 ± 2.9	27.7 ± 2.6	29.0 ± 3.3
	Female	20.7 ± 1.1	21.1 ± 1.4	22.3 ± 1.8
	All	22.9 ± 3.0	23.7 ± 3.8	25.0 ± 4.2
G93Ased	Male	24.7 ± 2.0	25.0 ± 1.6	24.1 ± 1.6*
	Female	19.9 ± 2.2	19.9 ± 2.2*	20.4 ± 2.3*
	All	23.0 ± 2.7	22.9 ± 3.7	22.6 ± 3.0*
G93Aex	Male	22.3 ± 1.1	22.1 ± 1.5*†	21.2 ± 1.7*†
	Female	19.5 ± 0.9	19.7 ± 1.3	19.3 ± 1.8
	All	20.9 ± 1.9	20.8 ± 1.8*	20.1 ± 2.0*†

WT = age-matched wild-type control; G93Ased = transgenic mice confined to normal caged activity; G93Aex = transgenic mice that underwent 8-weeks of treadmill running starting at 8 weeks of age. * indicates significant difference ($p<0.05$) from WT controls; † indicates significant difference ($p<0.05$) between G93Aex and G93Ased mice.

Table 2 **Average number of motor neurons displaying ChAT and FG staining 24-hr post-injection. Data are reported as mean ± standard deviation.**

Group	N	#ChAT+	#FG+
WT – exercise	8	92.8 ± 12.2	74.4 ± 8.7
WT – sedentary	8	98.6 ± 12.6	76.6 ± 10.2
G93A – exercise	9	76.8 ± 12.8*	44.1 ± 8.1*
G93A – sedentary	10	75.7 ± 17.7*	36.8 ± 9.7**

G93A (affected) vs. wild-type (control) mice organized by level of physical activity. The number of motor neurons reported reflects the number counted from 8 sections per animal. N= number of animals analyzed per group. There was a significant effect of disease status on the number of ChAT- and Fluoro-Gold-positive motor neurons counted ($p<0.01$). There was a significant effect of exercise on the number of Fluoro-Gold-positive motor neurons counted ($p<0.05$). The symbol (*) indicates significant difference from wild-type; (**) indicates significantly different from G93Aex.

Table 3 **Average number of neuromuscular junctions scored as innervated, intermediate, and denervated. All values reported as mean ± standard deviation.**

Group (N)	#NMJ counted	#Innervated	#Intermediate	#Denervated
WT (5)	200 ± 9	181 ± 6	13 ± 3	6 ± 2
G93Aex (5)	241 ± 22	102 ± 21	46 ± 9	93 ± 13
G93Ased (5)	208 ± 29	66 ± 17	37 ± 8	106 ± 13

WT vs. G93Aex vs. G93Ased mice. Five longitudinal sections of the medial right gastrocnemius muscle were counted per animal. The average number of neuromuscular junctions counted per group across five sections is shown. The average number scored as innervated, intermediate, or denervated per group is also shown.

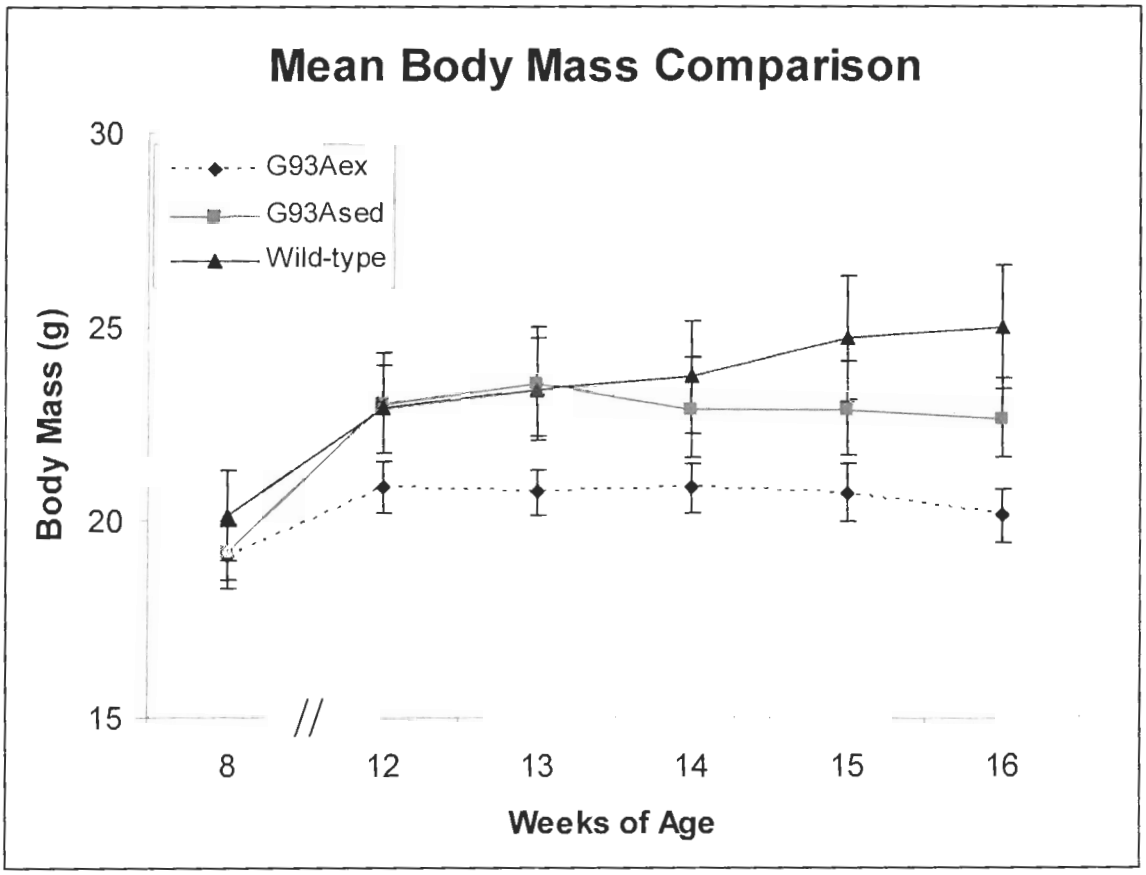

Figure 6 **Mean body mass comparison. All values reported as mean ± standard error.**

Wild-type (control) mice vs. G93Aex (affected exercise) vs. G93Ased (affected sedentary) mice. Data is sex-matched with same number of male and female mice per group.

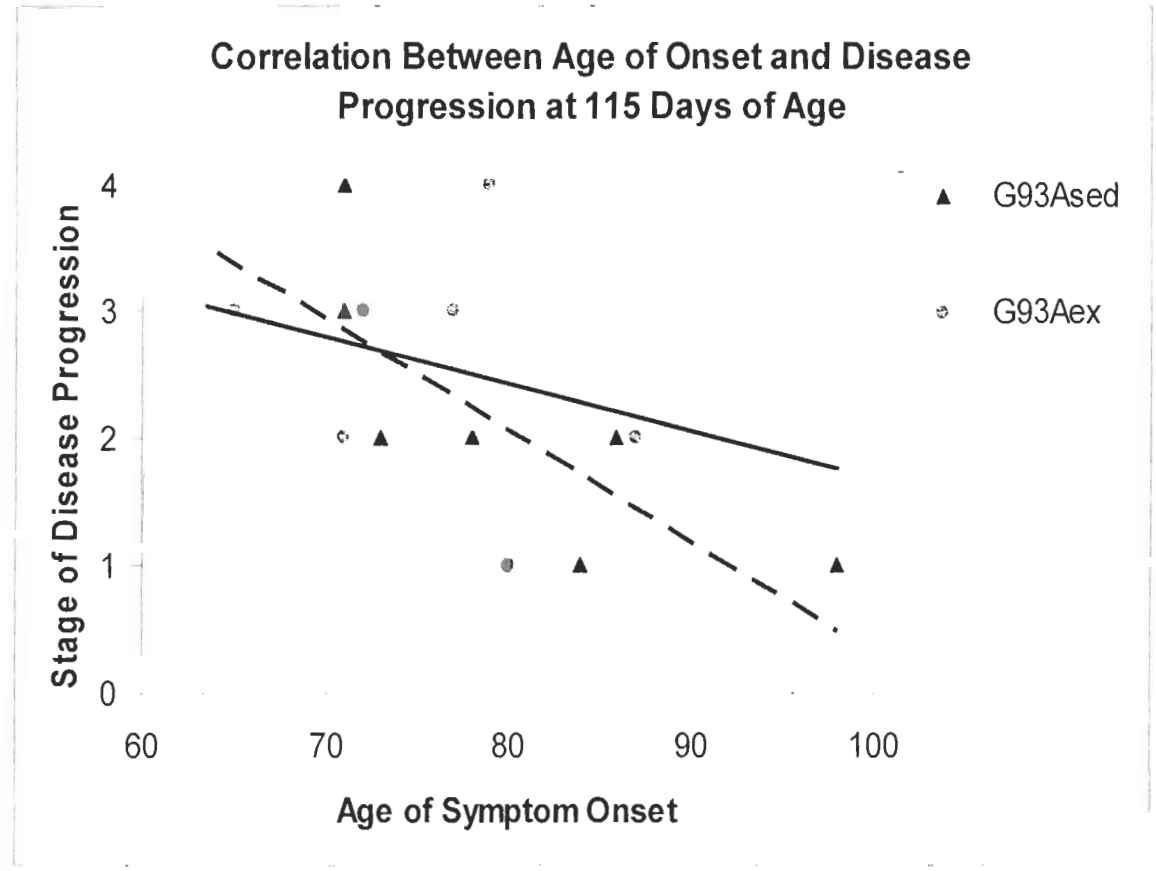

Figure 7 **Effect of exercise training on the relationship between age of onset and stage of disease progression in G93A mice.**

The stage (1-moderate, 2-late moderate, 3-advanced, 4-end-stage) that individual affected mice progressed to before sacrifice at 115 days is compared to age of symptom onset, as described in Appendix A. Age of symptom onset correlated well with stage of disease progression in sedentary G93A mice (black triangles, dotted line, $r = -0.80$, $p<0.05$). Mice who underwent endurance exercise training did not show a strong correlation (grey circles, solid line, $r = -0.32$, $p=0.49$) indicating exercise may have affected the rate of disease progression in some mice. However, analysis of covariance (ANCOVA) showed no significant difference in the slopes of the regression lines ($F=0.67$, $p=0.43$).

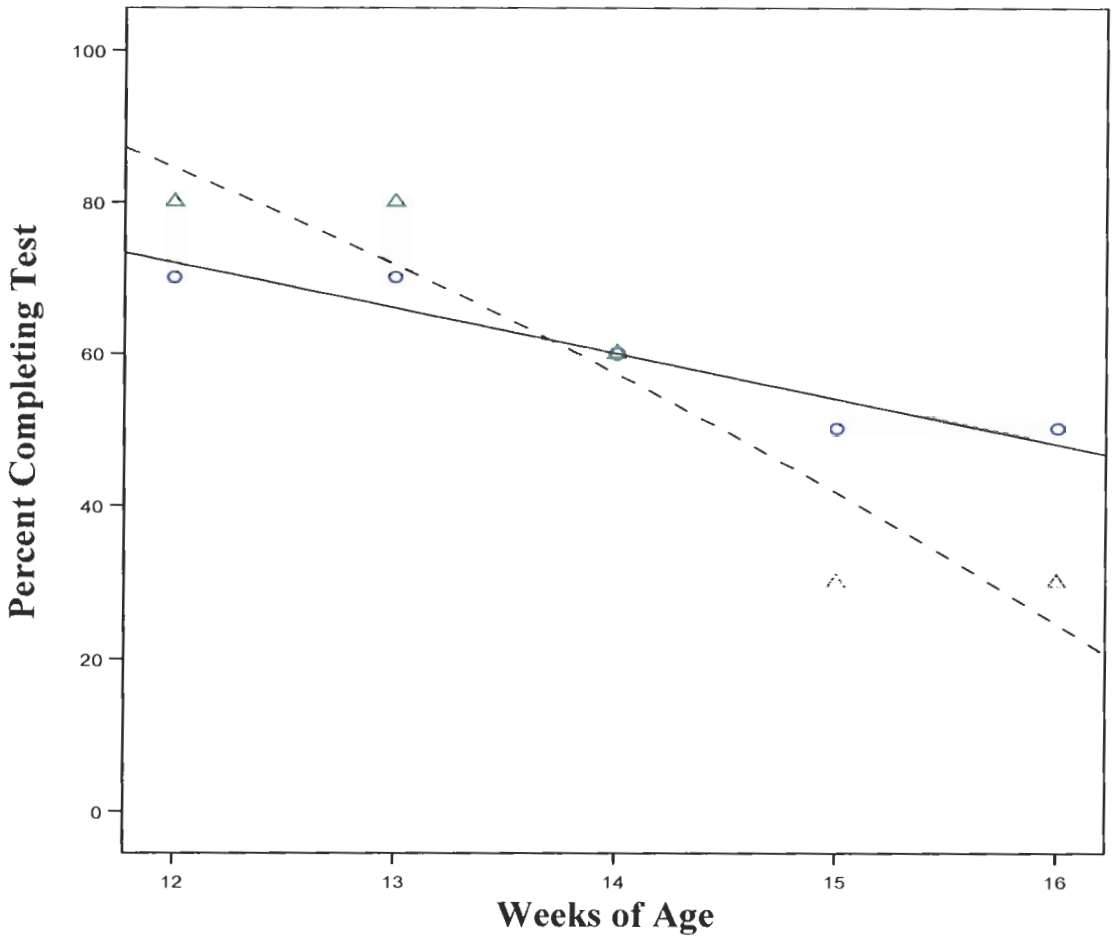

Figure 8 **Effect of exercise on rotorod performance in G93A mice. n=10 mice per group.**

Percent of mice able to complete the rotorod test without failing (160-180 seconds). In G93Ased mice (triangles, dotted line), only 30% of mice could complete the rotorod protocol without falling off prematurely, whereas 50% of G93Aex mice (circles, black line) could complete the test at 15 and 16 weeks of age.

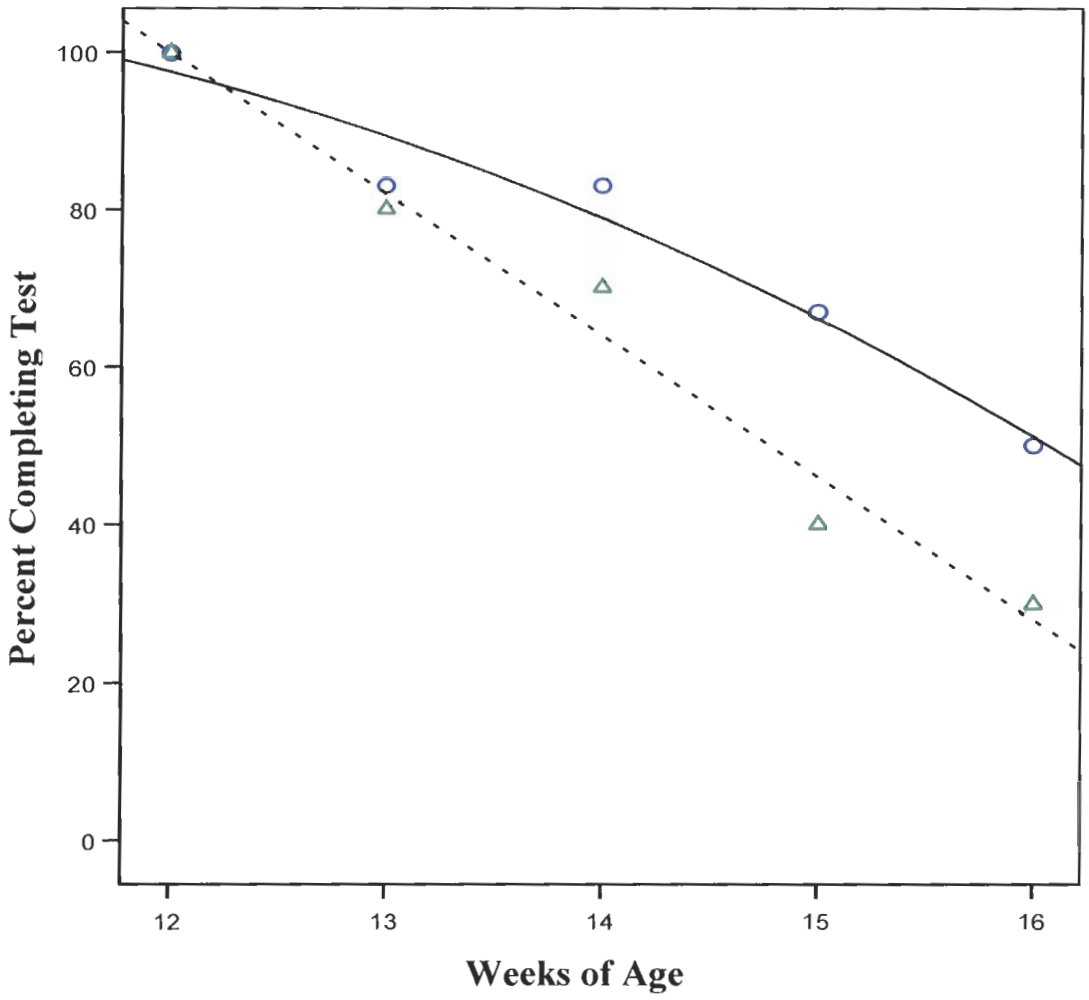

Figure 9 Effect of exercise on paw grip strength endurance (PaGE) test in G93A mice. n= 6-8 mice per group.

Percent of mice able to complete the PaGE test without failing (70-90 seconds). In G93Ased mice (triangles, dotted line), only 30% of mice could complete the rotorod protocol without falling off prematurely, whereas 50% of G93Aex mice (circles, black line) could complete the test at 16 weeks of age.

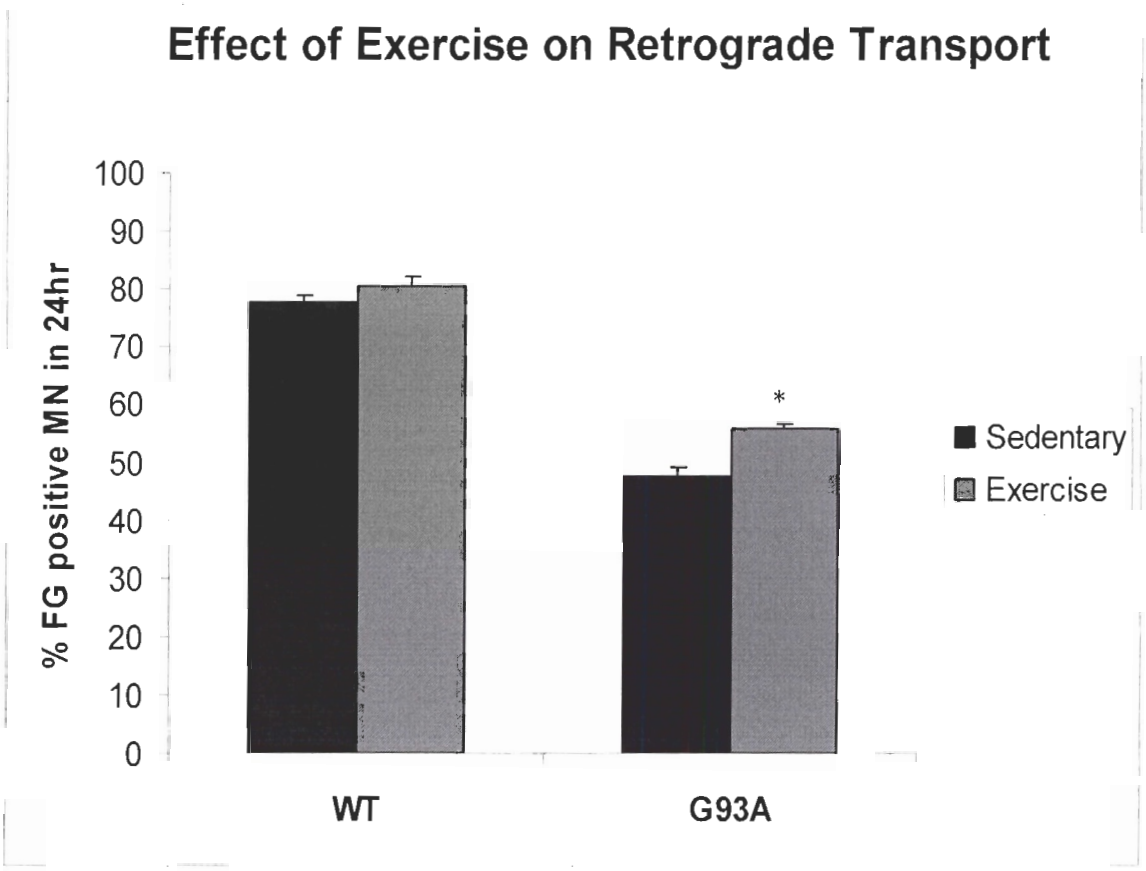

Figure 10 **Effect of exercise on retrograde transport rates in G93A and WT mice. All values are mean ± standard error, n= 8-10 per group.**

Mean percentage of lumbar motor neurons displaying Fluoro-Gold 24-hour after injection into gastrocnemius muscle. Affected G93A mice show significantly less Fluoro-Gold staining compared to control (WT) mice ($p<0.001$). Exercise significantly increases amount of MN displaying Fluoro-Gold in G93A mice ($p=0.001$).

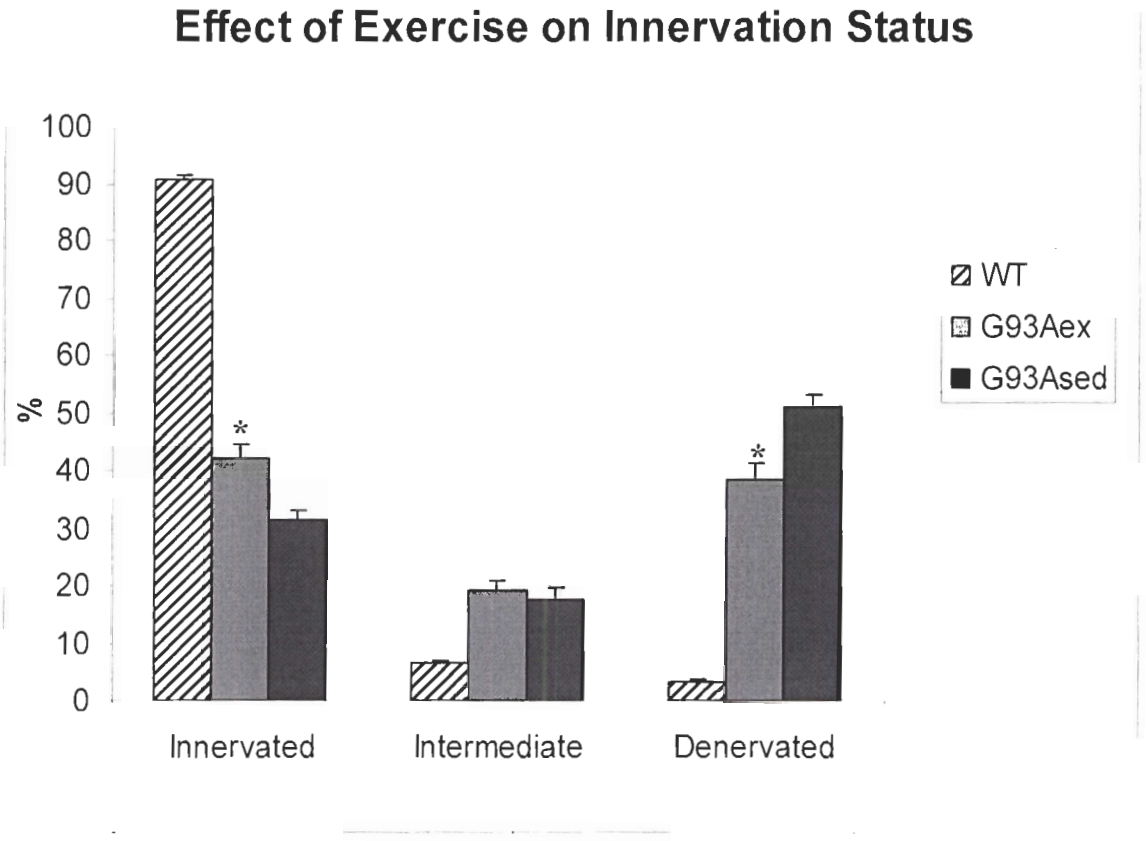

Figure 11 **Effect of exercise on mean percentage of NMJs characterized as innervated, intermediate, or denervated in G93A mice.**

The mean percentage of neuromuscular junctions counted and scored as innervated, intermediate, or denervated are shown for WT, G93Aex, and G93Ased mice. Innervated fibers showed complete overlap of terminal branches and neuromuscular junctions. Intermediate fibers showed axons in the vicinity of neuromuscular junctions, suggesting either acute denervation or possible reinnervation. Denervated fibers were not associated with terminal branches or axons (see Figure 4). The junctions in WT mice were almost completely innervated, and were significantly different from affected G93A mice in all categories ($p<0.001$). The asterisks (*) designate where G93Aex mice were significantly different from G93Ased mice ($p<0.01$).

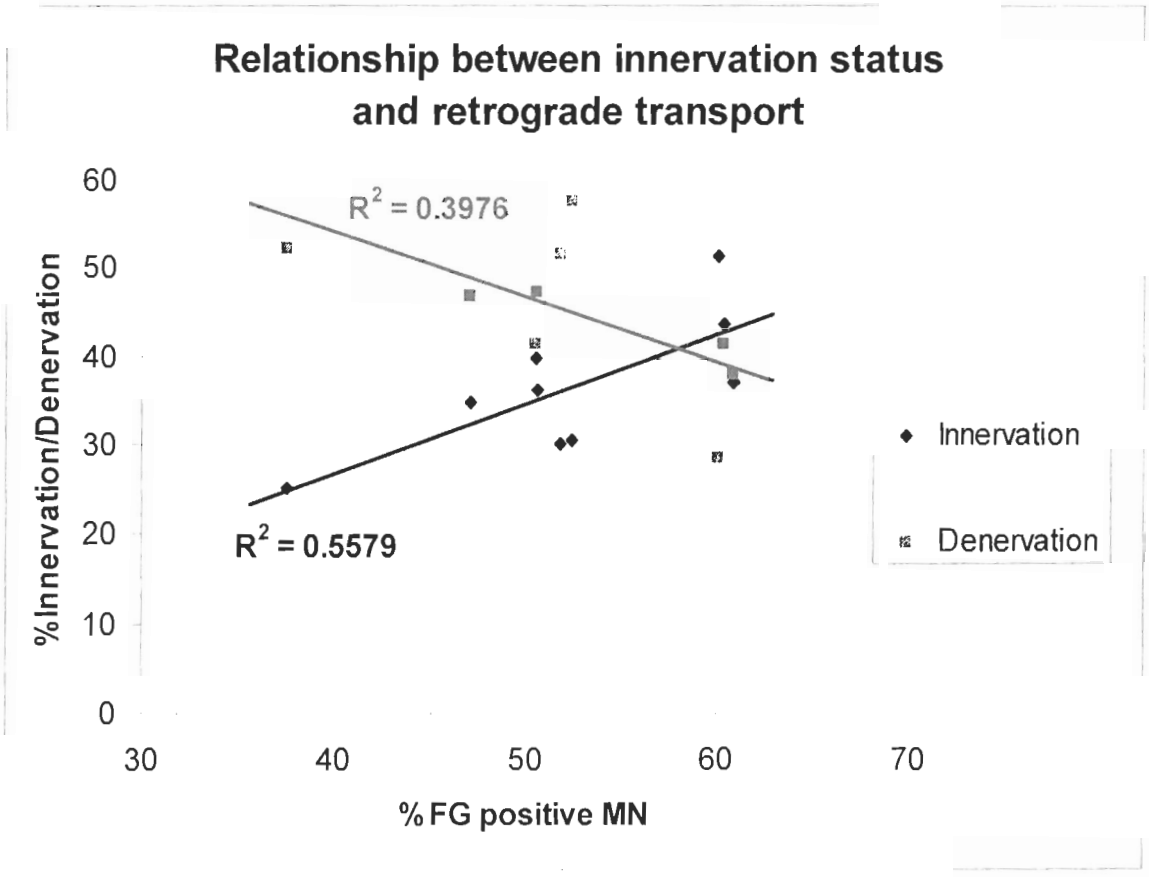

Figure 12 **Correlation between innervation status and retrograde transport of Fluoro-Gold.**

Retrograde transport rate correlated significantly with the percentage of innervated fibers at the neuromuscular junction in G93A mice (black line, $r = 0.75$, $p<0.01$). There was also a significant negative correlation between retrograde transport rate and the percentage of denervated muscle fibers (grey line, $r = -0.63$, $p<0.05$).

2.6 Discussion

Recent studies have shown endurance exercise training has the potential to significantly improve survival in a mouse model of amyotrophic lateral sclerosis (ALS). One study showed a dramatic 44-day improvement in survival following exercise alone, and an even greater 83-day increase by combining exercise and injection of the neurotrophic factor IGF-1 (Kaspar et al., 2005). However, few studies have explored the physiological mechanisms by which exercise could provide these benefits. The major finding of my study is that exercise improves retrograde transport in the mSOD1^{G93A} mouse model of ALS. This finding may provide one explanation for the improvements in survival and motor function seen following endurance exercise training in this mouse model.

To investigate these mechanisms, I chose to examine the retrograde transport of a neurotracer in the sciatic nerve of the mSOD1^{G93A} mouse following an 8-week exercise-training program. Animals performed treadmill running for 40 minutes per day, 5 times per week beginning at 56 days of age. The neurotracer Fluoro-Gold was injected into the gastrocnemius muscles of exercise-trained and sedentary G93A mice and their age-matched wild-type control animals. The animals were sacrificed 24 hours later, and the number of Fluoro-Gold labelled motor neurons in the lumbar spinal cord, identified by criteria set by Mojajeri and colleagues (1998), were counted and taken as a percentage of ChAT-positive cells. This provided an index of the number of surviving motor neurons able to take up Fluoro-Gold in 24 hours, which can be considered an indication of retrograde transport rate. As hypothesized, I found that retrograde transport rates were higher in G93A mice that underwent exercise training compared to sedentary controls. I

also examined the neuromuscular junctions of the injected muscles and found a higher number of innervated muscle fibers in the exercise group. These results suggest that one explanation for improvements in survival seen following exercise in this mouse model of ALS could stem from improvements in communication between motor neurons and muscles due to activity-dependent improvements in innervation.

G93A mSOD1 mouse – a model of ALS

Transgenic mice overexpressing mutant SOD1 protein develop an adult-onset progressive neuropathy, and have become the most widely accepted and used model of human ALS. Although only 2% of ALS cases result from mutations in the SOD1 protein, these transgenic mice currently provide the best starting point for the study of human ALS as they follow similar disease pathology (Gurney, 2000). The mSOD1^{G93A} transgenic mouse used in this study is a well-characterized model commonly used because of its short life span (~130 days of age) compared to other models such as the mSOD1^{G85R}, which survives up to a year (Subramaniam et al., 2002). However, the time from symptom onset to end-stage is the same in both of these models (Pun et al., 2006). One could argue that the G85R mouse more closely resembles human ALS as it is an adult onset disorder, whereas the G93A mouse is affected during development.

The average age of symptom onset, as defined by hindlimb flexion upon lifting by the tail, in the current study was 81 ± 11 days. This corresponds with data previously collected in our laboratory (Cunningham, 2005; McFee, 2005), but is slightly lower than the ~90 days previously seen in the literature (Chiu et al., 1995; Gurney et al., 1994; Mahoney et al., 2004). A possible explanation for this result could be that the transgene copy number was increased with breeding due to an unequal crossover event within the

transgene locus occurring during meiosis (Chiu et al., 1995). The considerable variation seen in age of onset in this study (65-101 days) could be accounted for by gender differences. Previous research has shown an earlier onset of clinical symptoms in male G93A mice compared to females (Veldink et al., 2003). The same result was observed in this study with average age of onset being significantly later in female mice than male mice (87 vs. 74 days, respectively; $p<0.01$).

At 84 days of age, there was no significant difference in the mean body mass of G93A mice versus age-matched wild-type mice. Wild-type mice continued to increase body mass up to 115 days of age, whereas G93A mice began to lose weight. At 115 days of age G93A mice had not lost a significant amount of weight compared to their baseline weight, but they did weigh significantly less than wild-type control mice. This observation conflicts with one previous study in our lab showing 20% loss of body mass from 85-115 days (McFee, 2005), but corresponds with another showing significant loss of body mass only from 122 days of age until death (Cunningham, 2005). Chiu and colleagues (1995) reported a 20% decline in body mass in G93A mice with most of the loss occurring after 115 days of age. One interesting finding from the current study was that G93A mice who underwent exercise training weighed significantly less than their sedentary controls throughout the exercise protocol. This was in agreement with endurance exercise studies in rats (Siu et al., 2004) and our mouse model (Mahoney et al., 2004) likely corresponding to a decreased fat mass or food intake. Although endurance exercise training does not typically result in muscle hypertophy, one goal of exercise training in this disease model would be to prevent denervation-induced muscle atrophy. There was potential evidence of this muscle sparing in the current study

suggested by the fact that sedentary male G93A mice lost more body weight from age 84 to 115 than did the mice that underwent endurance exercise training.

Exercise does not significantly improve motor function

There was a significant decrease in rotorod performance in G93A mice compared to age-matched wild-type mice beginning at 91 days of age, corresponding with the onset of symptoms. This finding was consistent with some previous studies (Derave et al., 2003; Mahoney et al., 2004), whereas others did not observe differences until after 115 days of age (Kaspar et al., 2005; Turner et al., 2004; Weydt et al., 2003). Exercise did not significantly improve average rotorod performance in G93A mice at any time point in the study. However, there were considerable variances within exercise and sedentary groups so I also looked at the percentage of animals able to complete the rotorod protocol at 16 weeks of age. Here I found 50% of G93Aex mice and only 30% of G93Ased mice able to complete the test indicating exercise may have influenced motor function in some animals. This is supported by a recent study conducted by Kaspar and colleagues (2005) that showed significant improvements in rotorod performance following endurance exercise training. Conversely, another study showed detrimental effects of exercise on rotorod performance, but this was likely due to the high intensity of their exercise training protocol (Mahoney et al., 2004).

Paw grip strength endurance (PaGE) was significantly diminished on average in both G93Aex and G93Ased compared to age matched wild-type mice at 16 weeks of age. G93Ased mice performed significantly worse than wild-type beginning at 14 weeks of age, a finding consistent with previous literature showing decline at 14 and 15 weeks of

age, respectively (Turner et al., 2004; Weydt et al., 2003). On the other hand, G93Aex mice did not perform significantly worse than wild-type until 16 weeks of age, suggesting exercise improved muscle strength-endurance in these mice. It was also determined that 50% of G93Aex mice compared to 30% of G93Ased mice could complete the PaGE testing protocol at 16 weeks of age. Although none of the other exercise studies in this mouse model measured grip strength-endurance, one study showed delayed decline in absolute grip strength in G93A mice that underwent exercise (Liebetanz et al., 2004). Exercise could improve grip strength and grip strength-endurance through maintenance of muscle innervation. Although endurance exercise training would target slow-twitch muscle fibers, muscular strength could be maintained longer into disease progression since motor units are recruited in a ramp-like fashion beginning with the slower motor units.

Exercise improves retrograde transport of fluorochromes

The major finding of this study is that exercise increased the percentage of motor neurons displaying Fluoro-Gold staining 24-hours post injection indicating improved retrograde axonal transport. In this mouse model of ALS, retrograde transport is diminished as early as 55 days of age (McFee, 2005), and continually declines throughout disease progression (Ligon et al., 2005). At my artificial endpoint of 115 days of age, G93Ased mice displayed Fluoro-Gold in 47% of motor neurons corroborating previous findings in our laboratory (McFee, 2005). This percentage was improved to 56% of motor neurons in G93A mice who underwent eight weeks of endurance exercise training. The total number of motor neurons counted was the same for the exercised and sedentary mice indicating no improvement in the survival of motor neurons. While this is in

agreement with the results of a study that used a similar treadmill running protocol and found no difference in motor neuron counts between exercise and sedentary G93A mice (Veldink et al., 2003), another study showed significant improvement in motor neuron survival following ad-libitum access to running wheels (Kaspar et al., 2005).

When comparing total surviving motor neurons (ChAT-positive) in G93A and wild-type mice, I observed a 23% loss at the 115 day time point. This is in agreement with previous data from our lab showing 18% loss of MN at 115 days, which then progressed to a 34% loss at 130 days near the end stage of disease (McFee, 2005). Although the loss was less severe than previously reported in the literature at end-stage (Chiu et al., 1995; Kaspar et al., 2005), the rapid decline in MN numbers in this disease model appears to occur sometime between 115 days and death (McFee, 2005; Mohajeri et al., 1998). This rapid loss has been shown to follow a period of stabilization with only gradual loss of MN during disease progression in mSOD mice (Feeney et al., 2001). The less severe loss observed might also reflect the fact that we did not differentiate between alpha and gamma motor neurons in this study since the smaller gamma motor neurons are resistant to disease (Mohajeri et al., 1998).

When counting the average number of Fluoro-Gold-positive motor neurons in G93Ased and wild-type mice, I observed a 52% reduction at 115 days indicating impaired retrograde transport mechanisms. This was similar to previous data in our lab showing 51% reduction at the same time-point (McFee, 2005). This result was not as severe as in a previous study using similar methods (Ligon et al., 2005), however that study also showed a significant decline in grip strength beginning at 70 days of age that was not observed in my experiment. Regardless of this discrepancy, previous studies are

in agreement that the reduction in Fluoro-Gold labelled cells is not due to a complete block in uptake or transport. This is evidenced by studies where increasing survival period post-injection to 72 hours completely abolishes any differences in retrograde labelling due to disease (McFee, 2005; Ligon et al., 2005). Ligon and colleagues (2005) suggested that the reduction in Fluoro-Gold-labelled cells at 24 hours was due to SOD1 colocalization with the molecular motor dynein causing disruptions in dynein-dependent retrograde transport. However, it cannot be ruled out that uptake at the neuromuscular junction is also slowed due to denervation of muscle fibers, which is a very early event in the disease process (Fischer et al., 2004).

The exercise-induced improvement in retrograde labelling is a novel finding in this disease model. Exercise has been shown to improve survival in this mouse model of ALS through yet unknown mechanisms (Kaspar et al., 2005; Kirkinezos et al., 2003; Veldink et al., 2003). Previous studies have linked endurance exercise training to improvements in the anterograde transport rate of various proteins in healthy rats (Beaumont and Gardiner, 2003; Gharakhanlou et al., 1999). My observation of improved retrograde transport following exercise in the G93A mouse may corroborate a recent study that combined exercise with retro-viral delivery of the neurotrophic factor IGF-1 (Kaspar et al., 2005). The synergistic effect of exercise and IGF-1 injection led to the biggest improvement in survival seen so far in this mouse model, suggesting that exercise improved delivery and/or uptake of the drug. This improvement could be due exercise-induced modulation of IGF-1 binding proteins (IGFBPs) resulting in improved signalling of downstream targets such as Bcl-xL, Bcl-2, and Akt. However, it cannot be ruled out that exercise is acting at the neuromuscular junction since IGFBPs have been implicated

in neuronal regeneration (Barnard and Aronson, 2005). The improvements in retrograde labelling with Fluoro-Gold following exercise could be due to improved uptake at the neuromuscular junction.

Exercise improves innervation status at the NMJ

I counted the number of innervated, intermediate, and denervated neuromuscular junctions in sedentary G93A mice and those that underwent exercise training. The G93Aex mice had a significantly higher percentage of innervated junctions than the sedentary group. These results suggest that exercise either preserved existing neuromuscular innervation or resulted in activity-induced sprouting and reinnervation of denervated fibers. Results for sedentary G93A mice showing 31% innervation at 115 days are consistent with previous literature showing ~33% innervation at 120 days of age (Fischer et al., 2004). It is important to note that in the Fischer (2004) study, denervation was much higher at 100 days than 120 days suggesting a period sprouting-induced reinnervation in an attempt to compensate for the loss. In the current study it appears that exercise promoted reinnervation. The level of innervation was higher than observed in the presymtomatic 80-day-old mice in the Fischer study.

It is possible that physical activity improved the sprouting response to denervation seen in this mouse model. Previous literature has shown that slow motor units in particular grow in the number of muscle fibers they innervate in response to the characteristic muscle denervation seen in this disease (Pun et al., 2006; Schaefer et al., 2005). However, eventually even these largely unaffected motor neurons are unable to compensate for the loss and the muscle becomes paralyzed near the endstage of disease. Some research has shown that physical activity improves the sprouting response in

denervated muscle (Einsiedel and Luff, 1994; Gardiner et al., 1984; Seburn and Gardiner, 1996), while other studies have shown it to be detrimental (Love et al., 2003; Tam and Gordon, 2003b). However, in studies where physical activity was detrimental, nerve transection was used causing acute denervation of up to 80% of muscle fibers. In studies where physical activity improved the sprouting response, muscle was only moderately denervated (~50% or less). In the current study, mSOD mice are a model of progressive denervation so muscles had likely undergone only moderate denervation at any time before the 115-day artificial endpoint.

The exercise training protocol began at 56 days in this study, just after the time-point where the first significant drop in innervation was reported by Fischer and colleagues (2004) at 47 days. This allowed for a long period of time where exercise could influence the sprouting and reinnervation response. The moderate-intensity endurance training protocol particularly recruited slow-twitch motor units, which have been shown to be resistant to disease and heavily involved in the compensatory sprouting response in mouse models of ALS (Schaefer et al., 2005). It has also been shown that treatment with ciliary neurotrophic factor (CNTF) alleviates the selective vulnerability of axons associated with fast-fatigable motor neurons leading to improvements in survival time (Pun et al., 2006). Exercise may also work to maintain these axons through upregulation of CNTF, although this has not been investigated. However, exercise has been shown to increase levels of various other neurotrophic factors such as IGF-1, BDNF, and NT-3.

I hypothesized that the exercise-induced improvements in retrograde transport of Fluoro-Gold would be related to improvements in the level of innervation at the

neuromuscular junctions of the injected medial gastrocnemius muscles. Indeed the results of this experiment were able to show increases in both the percentage of motor neurons displaying Fluoro-Gold and the percentage of innervated neuromuscular junctions. A Pearson correlation revealed a strong relationship ($r = 0.75$) between retrograde transport and level of innervation in G93Ased and G93Aex mice. This indicates that much of the improvement in retrograde transport was likely due to increased innervation and therefore improved uptake mechanisms occurring at the distal portion of axons. However, there must also be other factors involved in the process of retrograde transport that were improved by exercise.

Previous research has suggested that reduction in retrograde transport in diseased mice could also be due to alterations along the axon. In the *pmn* mouse model of progressive motor neuropathy retrograde transport of substances applied to the transected sciatic nerve was slowed suggesting deficit somewhere in the axon (Sagot et al., 1998). The actual speed of retrograde transport of *lacZ* was shown to be severely slowed in G93A mice compared to controls (Murakami et al., 2001). This could be due to mSOD1-mediated disruption of the dynein complex responsible for driving retrograde transport (Ligon et al., 2005), or the neurofilament inclusions seen along axons in this disease (Collard et al., 1995). It is quite possible that exercise, which has been shown to increase down-stream targets of neurotrophic factors in the spinal cord, also increased levels of these factors in the axons. This would allow for protection from the mSOD1 activity that is thought to damage motor axons in this disease model.

Effects of exercise intensity

Prior to the start of this study there had been three previous exercise studies in the G93A mouse model of ALS looking at survival time. The effects of exercise appeared to be intensity dependent with low-intensity endurance exercise improving survival (Kirkinezos et al., 2003), moderate-intensity exercise having no effect (Veldink et al., 2003), and high-intensity exercise decreasing survival (Mahoney et al., 2004). I therefore designed my study to use low-to-moderate intensity treadmill exercise since it had been shown to improve survival by 10 days in these mice. I determined that the treadmill speed I had chosen would correspond to about 75-80% of VO2max. It is likely that this lower intensity exercise was most beneficial in improving survival in the previous studies, and retrograde transport and innervation in the current study, because of recruitment and activation of slow-twitch motor units. The slow motor units have been shown to be resistant to disease and are highly involved in compensatory sprouting (Pun et al., 2006; Schaefer et al., 2005). Training the aerobic system likely allowed for improvements in the sprouting response of slow motor units and maximizing the number of muscle fibers remaining innervated. In contrast, the high intensity exercise training that had been shown to decrease survival in transgenic mice probably over-stressed the already compromised fast-fatigable motor units that are well known to be primarily targeted in this disease. Increased activation of these motor units likely leads to higher levels of oxidative damage due to free radical production and calcium turnover.

A more recent study showed that exercise at very low intensity for long durations on running wheels resulted in the most efficacious treatment for G93A mice to date (Kaspar et al., 2005). These findings were in contrast to another wheel running study

where relatively low exercise intensities produced no significant improvements in survival (Liebetanz et al., 2004). It seems the difference came from allowing the mice access to wheels but not forcing them to run at a certain pace in the Kaspar study. Although the mice covered a much more distance per day (up to 5 kilometers) in the Kaspar study than any of these other studies, it was covered over a 12-hour period where the mice were given ad libitum access to the running wheels. The mice were running at a lower relative intensity that was primarily stressing the aerobic system and slow motor units. Forcing mice to run at a predetermined pace in the current study and the others likely pushed the mice beyond there aerobic capacity to where the anaerobic system and fast motor units were stressed. Placing additional stress on the already compromised motor units could exacerbate the disease. Therefore, long duration exercise of low-intensity that is chosen by the mice is most effective in increasing survival of G93A mice.

Effects of gender

It is known that there is increased incidence and earlier age of onset of human ALS in men compared to women (Sorenson et al., 2002; Haverkamp et al., 1995). An earlier age of onset for male transgenic G93A mice was observed in the current study in agreement with previous literature (Veldink et al., 2003). The effects of exercise in G93A mice also appear to be sex-dependent. Two studies linking exercise to improvements in survival of transgenic mice found greater improvements in male mice (Kaspar et al., 2005; Kirkinezos et al., 2003). The current study has shown a significant effect of gender on the exercise-induced improvements in retrograde transport with only males showing significant improvement. Exercise also led to improved maintenance of body weight in male transgenic mice only. It has been suggested that the benefits seen in male mice

could be due to the effects of increased testosterone with exercise. Testosterone could provide neuroprotection of motor neurons through the action of androgen receptors (Brooks, 2000). However, it is possible that the greater improvement seen following exercise in male mice is because they are more severely affected at a given timepoint, so there is more room for improvement in neuromuscular communication.

In contrast, Veldink and colleagues (2003) found improved survival in female mice only following exercise training. They suggested this could be due to the neuroprotective effects of estrogen, which has been previously shown to increase following exercise above 70% of VO2max (De Crée, 1998). Estrogen also increases retrograde transport of Fluoro-Gold to lumbar motor neurons following sciatic nerve transection, where it is thought to work via the ERK and PI3K signalling pathways (Murashov et al., 2004). After age 60 the ratio of males to females with ALS is 1:1, compared to 4:1 in earlier decades of life, suggesting pre-menopausal women experience a protective effect of female sex hormones. More research is certainly warranted to determine the interactions of exercise and gender on ALS prognosis, particularly in regards to the potential therapeutic effects of different sex hormones.

Implications for viral vector therapy

Although there is no cure for ALS, recent research in the area of gene therapy with the use of viral vectors has been promising. Intramuscular injection of neurotrophic factors conjugated to adenoviruses allows for uptake, transport, and replication of these therapeutic agents in motor neurons. These treatments have prolonged life in mouse models of ALS, and have been shown to rely on adequate retrograde transport from muscle to nerve (Azzouz et al., 2000; Kaspar et al., 2003). The current study shows

exercise increases retrograde transport potentially via improved innervation of neuromuscular junctions. The combination of exercise and gene therapy produced a synergistic effect by increasing survival of G93A mice by 83 days (Kaspar et al., 2005). Taken together these results suggest exercise could improve the effectiveness of gene therapies by improving retrograde transport. Exercise-induced increases in sprouting could result in increased uptake and transport abilities of motor axons in mouse models of ALS (Millecamps et al., 2001).

One problem with the current gene therapies is that they are most effective when given early to presymptomatic mice. For human ALS patients this would not be possible since most patients do not realize they have the disease until after symptom onset. At this point, there is already motor neuron death and retrograde transport is significantly diminished. This may be an area where exercise, or some form of muscular stimulation, could improve the effectiveness of retroviral treatments via improvements in retrograde transport. Kaspar and colleagues (2005) showed that even late exposure to exercise, beginning after symptom onset (90 days), significantly improved survival in G93A mice. The combination of exercise and gene therapy after symptom onset still resulted in synergistic improvements in survival.

Future research

In order to further examine the potential role of exercise in treatment of ALS further studies must be done. Researchers could use the voluntary wheel-running exercise protocol from the Kaspar (2005) study to determine the role of retrograde transport in the improved survival of G93A mice following exercise. The use of a double-transgenic mouse (G93A-YFP) in future exercise studies would allow researchers to look at axonal

sprouting and subpopulations of motor units in-vivo at various time-points throughout the treatment protocol, as done previously in sedentary mice (Schaefer et al., 2005). Researchers could also transect the sciatic nerve and apply tracer to the stump to get a true measure of retrograde transport rate following exercise training. To determine if the effect of exercise on retrograde transport was due to sprouting in G93A mice, researchers could induce terminal axonal sprouting with the use of botulinum toxin and observe if retrograde transport is improved. To determine where in the axon exercise could be acting to improve transport, an exercise training protocol could be introduced to mice with dynein or neurofilament mutations to look for improvements in retrograde transport. Exercise could also be combined with gene therapy using other types of neurotrophic factors, such as CNTF, which is known to improve the health of axons.

Limitations

This study was limited by the number of mice I was able to exercise and analyze tissue from. With a large variation in age of onset and severity of disease before the time-point of sacrifice, some of the effect of the treatment may be due to individual differences in the groups. The study was also limited by the fact that I could only look at one time-point since there may have been a more dramatic effect of exercise on retrograde transport closer to the typical end-stage of the disease. The study was delimited by species and genetic factors. In terms of species, I used a mouse model of human ALS so the results of exercise can only be applied to mice and not humans with this disease. Similarly, I used the mSOD1^{G93A} transgenic mouse, but SOD1 mutations are only seen in approximately 2% of human ALS cases.

2.7 Conclusion

Results from this study demonstrate that exercise attenuates the reduction in retrograde transport in the G93A mouse model of ALS. This improvement appears to be highly related to an increase in the percentage of innervated neuromuscular junctions also seen following exercise in these mice. These results suggest that the improvements in survival shown previously with exercise training in G93A mice could result from increased neurotrophic support for motor neurons coming from skeletal muscle and/or improvements in the number of complete synapses. It is reasonable to assume that exercise was able to maintain the number of intact neuromuscular junctions as well as contribute to the sprouting response of compensatory slow-twitch motor units. Exercise may also have roles in improving the transport machinery in axons and the health of motor neurons directly through the upregulation of neurotrophic factors.

This study also provides further support for the use of exercise in combination with gene therapies because of their heavy reliance on effective retrograde transport. I speculate that exercise would need to be started early in the disease process to be most effective since denervation and retrograde transport disturbances are some of the earliest pathological consequences of this disease. In ALS patients where exercise is no longer possible, the effectiveness of these treatments could rely on electrical stimulation of the muscles and nerves. While this study failed to show definitive proof that exercise could improve motor function in this mouse model, further research should investigate the potential for exercise-induced improvements in neuromuscular function of ALS patients.

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APPENDICES

Appendix A: Summary of Disease Progression Characteristics

Normal Mouse -hind legs are extended and splay upon lifting from tail

Very Early -legs no longer splay upon lifting; reported as questionable

Early Signs -flexion in one or both hind legs upon lifting

-progresses from slightly flexed to fully flexed

-begin to see muscle atrophy evident at hips and hind legs

-rolling or paddling gait, especially evident when running on treadmill

-hunched posture

Moderate Signs -normally have reached maximal flexion of hind legs upon lifting

-hind foot knuckling when walking or running (drag toes of one or both hind legs

-develop a rough coat (usually only seen in males)

-increase in muscle atrophy

Advanced Stage -display partial or complete paralysis of one or both hind legs

-locomotion occurs via crawling or walking with entire body very close the ground

-start to lose balance at times and roll over

-some males show urine staining on coat

-weakness and atrophy can progress up through back muscles and even to front limbs

Endpoint -Need to count how long mouse spends in lateral recumbency when rolled over. Enpoint is lateral recumbency of 10 or more seconds.

Disease Progression Characteristics provided by Mary Dearden, Animal Care Facility, SFU

Appendix B: Immunohistochemical Staining Protocol

OCT Mounting

Spinal Cord

1. Place dry ice on metal working surface until surface is cold.
2. Apply Optimal Cutting Temperature (OCT) Medium to metal surface in the shape of a circle, about the size of a quarter. Surround area with dry ice cubes on all sides.
3. When circle of OCT is almost completely frozen, (a small spot of OCT in the middle should still be wet), place the caudal tip of the spinal cord into the wet center of the circle. This is done with atraumatic forceps being careful to squeeze spinal cord too hard.
4. Gently hold the spinal cord up until it freezes in place sticking upright.
5. Once the OCT has set and spinal cord stays in place, apply OCT around the spinal cord a small amount at a time in order to build up the mold in a pyramidal manner.
6. Repeat previous step, working around the cord and up until the entire cord is covered.
7. Gently remove the mold from the metal surface, wrap in parafilm and then tin foil and store at -80°C until ready to cut.

Muscle

1. Immerse bottom of the mold (tip of 50mL falcon tube) into liquid nitrogen to get cold. Fill bottom of mold with OCT medium (approx. ½ cm) and let it partially set.
2. Mount gastrocnemius muscle (previously quenched for 1 hour in glycine and quickly rinsed in 1X PBS) horizontally with the dorsal end placed in the unfrozen portion of the OCT mold. Place in liquid nitrogen until it freezes in place, then add more OCT and repeat until sample is covered.
3. After letting sample sit for approximately 1 minute, remove OCT mold from tube and wrap in parafilm and tinfoil for storage at -80°C until ready to cut.

Cryosectioning

Spinal cord

1. The cryostat should be set between –15 and –20°C and set to cut at 30μm.
2. Wipe down all surfaces with ethanol and check sharpness of blade.
3. Apply a small amount of OCT to the stage and then press sample into it. Allow it to freeze to secure sample to stage. Place in stage holder and line up with blade.
4. Before slicing sections set up a 4 X 6 well tissue culture plate by filling each well halfway with 1 X PBS.
5. Turn of safety on cryostat handle and begin slicing. Each spinal cord section should be picked up carefully with a paintbrush and placed into appropriate well.
6. Sections are put in order into successive wells of the plate so that every 4th section will be together in a row of the plate. The top row is typically used for the first trial of staining for each particular spinal cord.
7. Sections are able to be stored in the plate in 1 X PBS for up to 48 hours at 4°C. Extra sections not being used for staining right away can be stored in de Olomos solution in eppendorf tubes at –20°C until needed.

Muscle

1. Cryostat sections as follows: Section thickness: 40μm, box temperature: -23°C, block temperature: -17°C.
2. Mount mold onto stage with small amount of OCT, place stage onto block, and align sample with blade.
3. Cut through excess OCT and begin collecting muscle sections. Place poly-L-lysine coated slides into chamber approximately 30 seconds before mounting sections on the slide.
4. Collect every 5th section on one slide, and the next 5th section on another slide. One slide will be a back-up if staining needs to be repeated.
5. Once slides are full (6 sections per slide), immediately immerse in ice-cold acetone for 10 minutes for tissue post-fixation.
6. Place slides in rack to air-dry.
7. Proceed with IHC or store slides at 4°C until use.

Immunohistochemical Staining

SPINAL CORD[2]:

Procedure

1. Rinse sections 3 x 5 minutes in 1X PBS in 4 x 6 well tissue culture plate on shaker. Sections should be placed in small square containers with nylon netting that fit into wells so that solutions can be easily changed.
2. Prepare blocking solution (1mL per sample).
3. After removing 1X PBS, add 1mL of blocking solution to each well and incubate for 1 hour at room temperature on a shaker.
4. Prepare primary antibody solution (1mL per sample).
5. Remove blocking solution and add primary antibody solution to each sample. Place on shaker at room temperature for 1 hour.
6. Cover plate with parafilm and incubate at 4°C for 48 hours (24 hours is usually sufficient, but the longer sections can be left the better).
7. After removing primary antibody solution, rinse sections for 5 x 5 minutes in 1X PBS on shaker at room temperature.
8. Prepare secondary antibody solution (1mL per sample) in the dark with the use of foil.
9. Incubate in Secondary antibody solution for 1.5 hours at room temperature on shaker. Ensure to cover the plate with tin foil as the secondary is light sensitive. All remaining steps should be performed with minimal light exposure.
10. Rinse sections for 5 x 5 minutes with 1X PBS with 1X PBS.
11. Mount sections onto slides with a fine-tipped paintbrush.
12. Allow sections to air-dry, then add Vectashield Mounting Medium and coverslip.
13. Once Vectashield has dispersed evenly under coverslip, seal with clear nail enamel and let dry.
14. Store at 4°C in the dark.

Imaging[3]

1. Log into UV Lamp book—make sure to log hours used at start of session and hours on clock when finished.

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[2] Adapted with permission from the thesis of Ingrid McFee (M.Sc. 2005, SFU).
[3] Adapted with permission from the thesis of Lori Cunningham (M.Sc. 2005, SFU).

2. In the following order: turn on UV lamp, turn on camera, open MetaVue software program. (Reverse order when finished to turn off.)
3. Ensure shutter is closed and eye-piece view is selected (side of microscope).
4. Place slide under lens and select appropriate filter cube:

   U-MWU2 wide band cube filter: Fluoro-Gold (peak excitation: 360nm; emission at 620 – white). The visible light hitting the sections will appear blue; sections will immunofluoresce white.

   U-MWIG wide-band cube filter: Alexa Fluor 555 (peak excitation 555nm; emission at 565nm – red). The visible light hitting the sections will appear green; sections will immunofluoresce red.
5. Select the appropriate lens objective, focus slides through the eye-piece and then switch view to camera.
6. Through MetaVue program, select "Acquire", then "Acquire from Coolsnap" and set the following conditions:

   Acquire Menu: a) Select "Intensity Image"

   b) Adjust "Exposure Time" (1ms)

   Region Menu: Select "Entire Chip"

   Color Balancing: Select "Image Type" as "Fluorescence"

   Preferences: No need for adjustment
7. Select "Start Focusing"
8. Camera will be taking images every 100ms (selected exposure time). Adjust the following settings prior to acquiring final image:

   a) Select "pseudo color" on side menu bar of image – if you are imaging AF555 secondary, choose red; for Fluoro-Gold, choose blue.

   b) Acquire Menu: click on "Adjust Digital Contrast Icon"

   - adjust brightness and contrast to suitable levels.
9. Once image parameters are set, select "Stop Focus", then "Acquire Image"; close shutter. ***Note: Always ensure that shutter is closed when not in use – exposure to the UV light will fade signal over time.
10. For overlaying of a second antibody (e.g. AF555 and Fluoro-Gold), ensure that slide is not moved, and repeat steps 4-9 for the second antibody.

Image Overlay

1. On main menu, select "Display", then "Overlay Images"
2. In "Overlay Images Window": a) number of images should read 3

b) select correct source image for each color of overlay, e.g. Fluoro-Gold image for blue, AF555 image for red source

c) select "Apply Overlay"

Saving an Image

1. Save as TIFF file for use within MetaVue software.
2. Save a copy as an 8-bit TIFF to use within other software packages:

   -On main menu, select "Display", then "Scale Image", then copy to 8-bit

Solutions

20X Phosphate Buffered Saline (Stock solution, 250mL)

1. Make up the following solution in about 200mL with ddH_2O:

   -5.60g $NaHPO_4$ (Sodium Phosphate)

   -40.00g NaCl (Sodium Chloride)

   -1.02g KH_2PO_4 (Potassium Dihydrogen Orthophosphate)

   -0.96g KCl (Potassium Chloride)
2. Place on an electromagnetic stirrer and heat with while stirring to dissolve.
3. Once all salts have dissolved, feel the outside of the beaker. If hot, cool the solution to room temperature by placing beaker in cold water.
4. While stirring, adjust pH to 7.4 using 5 N NaOH.
5. Using a graduated cylinder, bring the total volume to exactly 250mL using ddH_2O.
6. Store at room temperature in the dark to preserve salts and prevent precipitation.

1X PBS Buffer (100mL)

1. Add 5mL of 20X PBS to 95mL of ddH_2O in a graduated cylinder.
2. Invert several times.
3. Check that pH is at 7.4, if not adjust using 1N HCl

Blocking Solution

0.1% Triton X

3% Normal Donkey Serum (or from animal secondary was raised in)

5% Bovine Serum Albumin

71.9% 1X PBS

To make 10mL, combine the following ingredients (needs to be vortexed thoroughly to mix completely):

10μL	Triton X
300μL	Normal Donkey Serum
500μL	Bovine Serum Albumin
9190μL	1X PBS

Primary Antibody

Choline Acetyl Transferase (ChAT)

- Chemicon International, AB144P, Goat X ChAT, Store at -20°C

Optimal dilution 1:200, dilute in blocking solution.

To make 1mL:

5μL	ChAT antibody
995μL	Blocking Solution

Secondary Antibody

Donkey Anti-Goat Alexa Fluor 555

- Molecular Probes (Invitrogen), A21432, store at 4°C in dark
- Optimal dilution 1:400, dilute in 1X PBS

To make 1mL:

2.5μL	Donkey anti-goat AF555
997.5μL	1X PBS

MUSCLE:

Procedure

1. Place filter paper on the floor of the humidifier and soak with water – ensure that humidifier is placed on an even surface.
2. Surround each muscle section with a PAP pen liquid blocker to prevent liquid run-off and preserve antibody.
3. Place slides in humidifier and re-hydrate sections by rinsing 3 x 5 minutes in 1X PBS.
4. Prepare desired amount of blocking buffer in 15mL falcon tube.
5. **Block for 1 hour** at room temperature. Be sure to decant all PBS by dabbing edge of section area with Kim-wipes (this assures blocking solution is not diluted by PBS).
6. Drain blocking buffer. Prepare primary antibodies at appropriate dilutions in 3-5mL test tubes (see solution recipes).
7. **Incubate in primary antibody solution for 24 hours at 4°C**. Be sure to cover humidifier in saran-wrap and be very careful to keep box level when transporting.
8. Rinse sections with 1X PBS (3 x 5 minutes).
9. Prepare secondary antibodies (in dark) in 1.5mL eppendorf tubes covered in tinfoil. Both secondary antibodies can be prepared as 1 solution.
10. **Incubate in secondary antibody solution for 90 minutes (in dark).** Be sure to cover humidifier in tinfoil for the remainder of the steps to prevent fading of the secondary antibodies.
11. Remove secondary antibody solution and wash with sections with 1X PBS (3 x 5 minutes).
12. Drain excess PBS, but add Vectashield Mounting Medium (a few drops per slide) before sections dry out.
13. Once Vectashield has dispersed evenly under cover slip, seal with clear nail enamel and let dry.
14. Store slides at 4°C in dark until imaged.

Imaging

1. In the following order, turn on UV lamp, turn on Leica CM400 microscope, and open Leica Application Suite software program.
2. Open shutter and press fluorescence button.
3. Place slide under appropriate objective (20X to find region, then 40X for counting).

4. Select filter cube: TxRed-4040B for neurofilament and SV2, FITC-3540B for NMJ.
5. Focus region through eyepiece then switch to camera by pulling out bare located next to the eyepiece. View using software and adjust exposure and focus.
6. Under "λ" tab an **overlay** can be taken by checking the boxes located next to FITC and TxRed.

Solutions

Blocking Solution

To make 2mL, combine the following ingredients (needs to be vortexed thoroughly to mix completely):

2μL	Triton X
20μL	Fab Fragment Goat Anti-Mouse IgG
60μL	Normal Goat Serum
100μL	Bovine Serum Albumin
1818μL	1X PBS

Primary Antibodies

Mouse Anti-Neurofilament (NF160)

- Chemicon International, MAB5254, Ms X Neurofilament, Store at 4°C
- Optimal dilution 1:400, dilute in blocking solution

Synaptic Vessicles

-SV2, Iowa Hybridoma Bank, Store at 4 °C

-Optimal dilution 1:100, dilute in blocking solution

To make 1mL:

2.5μL	Ms X Neurofilament
10μL	SV2
987.5μL	1X PBS

Secondary Antibodies

Cy3

-Cy3-conjugated AffiniPure Goat Anti-Mouse IgG, #115-165-146, Jackson ImmunoResearch Laboratories, Inc.

-Optimal dilution 1:200

FITC-α-bungarotoxin

-Fluorescein-α-bungarotoxin, #00011, Biotium, Inc.

-Optimal dilution 1:200

To make 1mL:

5μL	Cy3
5μL	FITC
990μL	1X PBS