EFFECTS OF EXERCISE ON NEUROPROTECTION IN EXPERIMENTAL

AUTOIMMUNE ENCEPHALOMYELITIS

by

WILLIAM MILTON PRYOR

(Under the Direction of Lesley J. White)

ABSTRACT

Multiple Sclerosis (MS) is an autoimmune disease affecting the central nervous system that results in continued loss of cognitive, sensory, and motor function. In this study we aimed to evaluate the effects of voluntary exercise on the hallmarks of neuropathology and activation of pro-survival pathways in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. C57BL/6J mice were injected with an emulsion containing myelin oligodendrocyte glycoprotein (MOG) and then randomized to housing with a running wheel or a locked wheel. EAE mice exposed to exercise displayed less severe neurological disease score and later onset of disease when compared to sedentary EAE animals. Immune cell infiltration and demyelination in the ventral white matter tracts of the lumbar spinal cord was significantly reduced in the EAE exercise group compared to sedentary EAE animals. Axon immunolabeling in the ventral pyramidal and extrapyramidal motor tracts displayed a more random distribution of axons and apparent loss of smaller diameter axons with a greater loss of immunolabeling in the sedentary EAE animals. In lamina IX grey matter regions of the

lumbar spinal cord, sedentary animals with EAE displayed a greater loss of α-motor

neurons when compared to EAE animals exposed to exercise. Phosphorylation of TrkB

receptors was significantly increased in the exercise group when compared to sedentary

EAE animals and was distributed throughout the ventral horn and α-motor neurons.

Expression of mitochondrial outer membrane pro-survival members Bcl-2, Bcl-XL, and

Mcl-1 were all significantly higher in the EAE animals exposed to exercise. These data

suggest that chronic voluntary exercise positively alters the autoimmune response and

activates intrinsic pathways that lead neuroprotection.

INDEX WORDS: Multiple sclerosis, Experimental autoimmune encephalomyelitis, Exercise, Neuroprotection

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CHAPTER 1

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease that acts on the central nervous system (CNS) and affects approximately 400,000 individuals in the USA and 2.5 million people worldwide (Peterson et al., 2001). MS is most often diagnosed in those between the ages of 20 and 40 with a male-to-female ratio of 1:2 and is the leading cause of neurologic disability in young adults (Ramagopalan et al., 2010; Richards et al., 2002). Approximately 10,000 new cases of MS are diagnosed annually in the USA (Grima et al., 2000).

Multiple sclerosis is a T-cell mediated autoimmune disease that primarily targets the myelin glycoproteins surrounding axons in the central nervous system (CNS) (McFarland and Martin, 2007). This results in the development of lesions that accumulate over time, and leads to loss of axonal processes and progressive loss of gray matter. It is this loss of the gray matter that leads to the progressive cognitive and physical disability that individuals with MS experience (Fisniku et al., 2008).

People that have MS may experience several types of defined disease courses in their lifetime (Sospedra and Martin, 2005). Most forms of the disease are punctuated by attacks of declining neurologic and physical function that are followed by partial or complete recovery periods. More aggressive forms of MS are characterized by increasing neurological dysfunction with no significant relapsing or remitting phases. People with MS experience a myriad of physical and mental symptoms that evolves into cumulative

and progressive disability, with a large percentage of people with MS eventually becoming wheelchair-bound (Richards et al., 2002).

Animal models of MS such as experimental autoimmune encephalomyelitis (EAE), have been used to delineate the mechanisms of cellular and molecular signaling pathways such as those activated by neurotrophins and growth factors. Neurotrophins bind to signal transducing tyrosine kinase receptor, the tropomyosin related kinase (Trk) receptors; which modulate function, survival, proliferation, differentiation, myelination, apoptosis, and axonal growth (Arevalo and Wu, 2006).

Exercise has been shown to promote neuroprotection in people with MS and animals with EAE (Castellano and White, 2008a; Rossi et al., 2009) through anti-apoptotic/pro-growth mechanisms such as upregulation of neurotrophin signaling (Liu et al., 2008a; Macias et al., 2007; Skup et al., 2002). Studies examining the effects of exercise in animal models have revealed that exercise activates mechanisms that lead to neuroprotection, delays dendritic spine loss, increases neural progenitor cell proliferation, and reduces overall clinical disease severity (Rossi et al., 2009; Le Page et al., 1996; Magalon et al., 2007). To date, no researchers have examined the impact of exercise on the regulation of apoptosis in the CNS of EAE animals.

Statement of the Problem:

Nearly half of a million people in the USA have been diagnosed with MS and almost 200 people are diagnosed weekly. MS is extremely costly to the individual with the disease, their family members, and the US healthcare system. The cost of MS has been estimated to be over $34,000 annually per person, with a total lifetime cost of $2.2

million per individual. A conservative estimate of the national annual cost of healthcare is $6.8 billion with most of the costs being absorbed by Medicare and Medicaid (Whetten-Goldstein et al., 1998). Over 64% of healthcare costs can be attributed to MS-specific prescription drugs, with the cost of over $20,000 annually for each individual with the disease (Prescott et al., 2007).

Although there is currently no cure for MS, there are treatments available that are aimed at modifying the disease course and managing related symptoms. These treatments are aimed at modifying the immune response, yet are only moderately effective and often have profound side-effects. Multiple sclerosis causes impaired mobility that inhibits or discourages activities of daily living and in turn heightens the risk for hypokinetic disorders such as coronary artery disease (CAD) (White et al., 2006; Slawta et al., 2003) and a progressive decline in neurological function (Kuhle et al., 2011). Exercise has been shown to preserve or enhance physical function and confer neuroprotection in people with MS (Heesen et al., 2006; Castellano and White, 2008b). Exploring the molecular basis of exercise in neuroprotection in an animal model of MS is important for determining the efficacy of exercise as a treatment for MS, as well as identifying the signaling pathways involved in exercise mediated neuroprotection for targets of future therapeutics.

Study Aims and Hypotheses:

This research will address the following specific aims:

Aim 1: To determine whether exercise attenuates the onset of disease and reduces the severity of EAE when compared to sedentary controls with EAE.

Rationale 1: Voluntary wheel running in EAE animal models has shown to be effective at attenuating disease onset and reducing severity in two other studies (Rossi et al., 2009; Le Page et al., 1996; Berchtold et al., 2005). Rossi and colleagues showed that exercise reduced overall clinical disease severity and neurological deficits compared to control animals after EAE induction (Rossi et al., 2009). EAE mice that have exposure to running wheels beginning at the time of EAE induction show delayed onset of disease and decreased disease severity as compared to sedentary EAE animals (Magalon et al., 2007). Exercise can also delay the onset of clinical signs of disease in adoptive transfer of EAE in mice forced to run on treadmills (Le Page et al., 1996). We will attempt to repeat these results and hypothesize that exercise may attenuate disease onset and ameliorate disease severity throughout the course of 25 days.

Aim 2: Determine if exercise has an effect on altering the amount of infiltrating immune cells from the periphery into the lumbar spinal cord.

Rationale 2: In EAE experimental animals, there are large numbers of infiltrating immune cells found in the CNS during the disease course (Choi et al., 2011; Wu et al., 2010). Rossi and colleagues examined the effects of exercise in the EAE model and showed that haematoxylin and eosin staining of the spinal cord revealed large alterations in both EAE and EAE exercising mice. Morphological alterations were observed in both gray and white matter of the spinal cord, however differences between the two groups were not observed, but their analysis was performed mainly on the striatum (Rossi et al., 2009) and not the lumbar spinal cord where T-cells gain entry to the CNS. Although exercise has been shown to delay the onset and reduce the severity of EAE, Rossi et al.

explains that autoimmune infiltration and inflammation continues to occur during an exercised condition and the neuroprotective properties of exercise are not related to an immune-mediated mechanism, but rather mechanisms that are intrinsic to the CNS (Rossi et al., 2009). Due to alteration in T-cell regulatory mechanisms and cytokine alterations with chronic exercise, we hypothesize that EAE animals exposed to exercise will have attenuated infiltration of immune cells when compared to the sedentary EAE animals.

Aim 3: Examine the effects of exercise on demyelination in the white matter tracts of the lumbar spinal cord.

<u>Rationale 3</u>: Myelin glycoproteins are the target of infiltrating activated immune cells from the periphery, which typically results in the loss of myelin in many regions of the CNS. Much of the pathophysiology of EAE has focused on the spinal cord, but immune cell infiltration, demyelination, neural apoptosis, and axonal loss occurs in the cerebellum and other brain regions (MacKenzie-Graham et al., 2009). Mice with EAE demonstrate motor and balance deficits that are indicative of damage to the corticospinal and spinocerebellar tracts. Mackenzie-Graham and colleagues measured significant decreases in the total volume of the cerebellum, and molecular layer of the cerebellar cortex in EAE mice with large expanses of demyelination in the cerebellar white matter (MacKenzie-Graham et al., 2009). Jackson et al used a chronic relapsing EAE model and discovered that demyelination occurred after each relapse and correlated with increasing residual motor deficits in remission. Subsequent lesions displayed significant evidence of demyelination, remyelination, axonal degeneration, and axon loss (Jackson et al., 2009). There have been no studies examining the effects of exercise on demyelination in the

EAE model as of yet. If chronic voluntary exercise is able to attenuate autoimmune cell infiltration into the lumbar spinal cord, we hypothesize that EAE animals exposed to exercise will exhibit preservation of myelin within the white matter tracts of the spinal cord.

Aim 4: Examine axonal loss in the anterior pyramidal and extrapyramidal descending motor tracts of the lumbar spinal cord.

Rationale 4: The corticospinal system is the only direct pathway from the motorsensory cortex to the spinal cord, and the major neural pathway for control of voluntary movement. It is also frequently involved in the pathological process of EAE and degeneration in these regions lead to motor disability. Evaluation of the corticospinal tract showed a high correlation between the axonal loss and the clinical disease severity score in the EAE mice (Liu et al., 2008b). The loss of axons in myelinated tracts is secondary to the loss of myelin and neural apoptosis that occurs in EAE and MS. Lovas et al evaluated axonal degenerative changes in autopsy spinal cords of humans that had been suffering from secondary progressive MS by using neurofilament immunolabeling. They observed a significant reduction in axonal density in the normal appearing white matter and within spinal cord plaque regions (Lovas et al., 2000). Tallantyre and colleagues obtained spinal cord sections from 45 people with MS during autopsy. Those who had accumulated higher levels of motor disability prior to death demonstrated fewer surviving corticospinal axons (Tallantyre et al., 2010). In a chronic relapsing-remitting EAE model, axonal loss was first evident during the acute phase of disease and axonal loss continued to occur after each relapse and correlated with increasing residual motor

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deficits in remission (Jackson et al., 2009). We hypothesize that, since exercise has been shown to preserve motor function in EAE animals, chronic voluntary exercise will preserve axon density in the ventral descending white matter tracts when compared to sedentary EAE animals.

Aim 5: Determine if TrkB receptors are upregulated and activated in the lumbar spinal cord gray matter in response to exercise in EAE animals.

<u>Rationale 5</u>: The level of phosphorylation of the kinase domains of the TrkB receptor is highly correlated with the availability of BDNF, which is a neurotrophin that is upregulated in response to exercise (Berchtold et al., 2005). Phosphorylated TrkB receptors are increased in the hippocampus after four weeks of exercise (Liu et al., 2008a) and ablation of TrkB receptors in neural progenitor cells can inhibit exercise-induced increases in hippocampal cell proliferation and neurogenesis (Li et al., 2008). Macias and colleagues found that TrkB mRNA was increased in the lumbar spinal cord ventral horn neurons and oligodendrocytes within the spinal gray matter in Wistar rats after 28 days of treadmill exercise. TrkB mRNA transcripts were also found throughout the dorsal horn, with a particularly high density in the superficial laminae (Macias et al., 2007). Other investigators have also confirmed the increases in TrkB receptors in the grey matter of the spinal cord after a period of exercise training, with the majority of the TrkB positive cells being oligodendrocytes (Skup et al., 2002). Exercise has been shown to promote increases in TrkB and phosphorylated TrkB, with no significant changes in the truncated gp95trkB receptor (Liu et al., 2008a; Skup et al., 2002). These observations suggest that CNS cells become more responsive to neurotrophic stimuli and more

resistant to apoptosis after exercise. These data lead us to hypothesize that EAE animals exposed to exercise group will show higher expression of TrkB receptors, and due to increases in BDNF during exercise, EAE animals that exercise will have increased activation of TrkB receptors.

Aim 6: Investigate downstream targets of TrkB signaling that are involved with inhibiting apoptosis.

Rationale 6: Apoptosis can be divided into two broad categories including the intrinsic and extrinsic pathways that often converge on each other at certain points in the signaling cascade. Both pathways result in the release of mitochondrial proteins such as cytochrome-c to activate the cleavage of caspases. Transduction of death signals at the mitochondrial checkpoint are BH3-only proteins which interact with the Bcl-2 family proteins to allow release of mitochondrial pro-apoptotic proteins (Putcha et al., 2003). The Bcl-2 family of proteins consists of a number of evolutionarily conserved sequences containing Bcl-2 homology domains (BH) that modulate apoptosis through mitochondrial membrane permeability and cytochrome-c release (Cory et al., 2003). The Bcl-2 family consists of three groups based upon homology and function. Pro-survival members include Bcl-2, Bcl-xL, and Mcl-1, and pro-apoptotic proteins include Bax, Bak and the BH3 only proteins such as Bad, Bik, Bid, Bim, Puma, and others. Interactions between pro-apoptotic and anti-apoptotic Bcl-2 family members control cells' fate by forming homo and heterodimers, with the ratio of pro to anti-apoptotic proteins determining death or survival (Datta et al., 1997). These pro-survival members exert anti-apoptotic activity by binding to and antagonizing the death-promoting members by ultimately inhibiting

mitochondrial pore formation and activation of caspases (Cory et al., 2003). These anti-apoptotic proteins can be stimulated by cytokines, growth factors, and neurotrophins. Neurotrophin deprivation activates the c-Jun N-terminal kinases (JNKs) that culminate in activation of the BH3-only BCL-2 proteins and the ultimate release of BAX-dependent cytochrome-c release, caspase cleavage, and apoptosis (Putcha et al., 2003). BDNF can promote cell survival by activating the TrkB receptor and tyrosine kinase signaling through phosphatidylinositide-3'-OH kinase (PI3K) and its downstream target Akt, a serine-threonine kinase. Akt phosphorylates proteins at the mitochondrial membrane and inhibits the cascade of events that lead to mitochondrial membrane permeability and ultimately, apoptosis (Datta et al., 1997). This pathway also leads to phosphorylation of caspases (Cardone et al., 1998) and suppresses the expression of death genes by phosphorylating the Forkhead box family of transcription factors (Biggs et al., 1999). Nguyen and colleagues found that BDNF prevents staurosporine induced apoptotic activity and caspase cleavage by upregulation of the phosphorylation of the TrkB receptor in hippocampal H19-7 cells. Inhibition of the Trk receptor by K252a abolished the protective effect BDNF, while impairment of the PI3K/Akt pathway abolished the protective effects of BDNF, and siRNA knockdown of Akt was also able to block the pro-survival effects of the neurotrophins (Nguyen et al., 2010). These data show that activation of the TrkB receptor and activation of the PI3K/Akt pathway confer an anti-apoptotic effect on neurotoxicity. No studies have examined the effects of exercise on regulating apoptosis in the CNS of the EAE animal model. As exercise increases BDNF expression, and *in vitro* studies show that ablation of this signaling pathway leads to cell

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death, we hypothesize that exercise can translationally increase expression of Bcl-2 family pro-survival members in EAE animals exposed to exercise.

Significance of the Study

Multiple sclerosis is the leading cause of neurologic disability in young adults. People with MS live with increasing disability, loss of independence, and reduced quality of life along with a high social and economic cost. Individuals typically develop the disease during the third or fourth decade of life, but often have a near normal lifespan. They however; live with increasing disability and in many cases lose their ability to function independently. Since there is no cure for MS, strategies to reduce disease progression may extend the health and quality of life of those with the disease. The proposed study will expand our understanding of how exercise may influence factors associated with disease progression in MS. The relevance of this work will help to determine whether exercise is a viable therapeutic approach to lessen the severity of EAE, which has striking similarities to MS. Data obtained in this study will provide further insight into the molecular nature of exercise as a therapeutic treatment for EAE, which there is currently little data. Study results will enable health care professionals to make informed decisions regarding patient care and will help to delineate possible molecular targets for future pharmacological intervention.

CHAPTER 2

REVIEW OF THE RELATED LITERATURE

Multiple Sclerosis: Epidemiology and Etiology

Multiple sclerosis (MS) is a complex, chronic autoimmune disease affecting the central nervous system (CNS). The disease affects approximately 400,000 individuals in the USA and 2.5 million people worldwide. MS most often affects those between the ages of 20 and 40 with a male-to-female ratio of 1:2 and is the leading cause of neurologic disability in young adults (Ramagopalan et al., 2010; Richards et al., 2002). Approximately 10,000 new cases of MS are diagnosed annually in the USA (Grima et al., 2000), yet for unknown reasons, the prevalence varies largely according to geographic areas (Compston and Coles, 2008; Noseworthy et al., 2000). There is increased prevalence in individuals that are Caucasian and of Northern European descent, and those living in temperate geographical areas (Grima et al., 2000). Regions that were settled by Northern European tribes and Vikings have the highest prevalence of MS throughout Europe, South Africa, Australia, and the New World. In Asia, Africa, and the Tropics, MS is quite rare and prevalence rises with increasing distance from the equator (Kurtzke, 1995). The incidence of MS is the highest at the extreme latitudes in the Northern and Southern hemispheres, and is related to ethnic differences such as Caucasians of Northern European descent (Visscher et al., 1977).

While the exact cause or trigger for MS is unknown, research indicates the etiology is likely due to complex interactions between genetics, infectious pathogens, or environmental exposure in susceptible individuals (Burks, 2005). Genetics have been shown to play a large role in susceptibility to MS. Multiple sclerosis is a polygenic disease, so identifying a single gene that leads to MS susceptibility has not been possible. The only confirmed gene that leads to an increased risk for MS is the human leukocyte antigen (HLA) DR2 allele (Kalman et al., 2002; Madsen et al., 1999). A few additional genes have also appeared to be involved in the development of MS, such as the IL-2Rα and IL-7Rα alleles (International Multiple Sclerosis Genetics Consortium et al., 2007). Although genetics play a crucial role in susceptibility to disease, there seems to be another trigger to the development of MS such as environmental factors. Identical twins should have the same incidence of disease, yet the concordance rate for monozygotic twins is only 25-30%. Although this relationship is high when compared to dizygotic twins and non-twin siblings, disease discordance in monozygotic twins has been interpreted to indicate environmental importance in MS pathogenesis (Baranzini et al., 2010).

Multiple sclerosis pathology has been explored in both noninfectious (smoking, sunlight, toxins) and infectious environmental factors such as Epstein-Barr virus (EBV), herpes virus, and others that may help to explain patterns of geographical variation (Ascherio and Munger, 2007). The risk of developing MS is approximately 10 times greater among people who had been infected by EBV, yet not diagnosed with mononucleosis during childhood. The risk increases more than 20 times among those who developed clinically diagnosed mononucleosis (Ascherio and Munger, 2007;

Serafini et al., 2007). EBV infects a large percent of the population, yet the period of time in which EBV is acquired along with genetic susceptibility may provide enough stimuli to develop an autoimmune response (Serafini et al., 2007). One of the greatest environmental risk factors for MS is the link between latitude and exposure to sunlight and subsequent vitamin D levels (Ascherio and Munger, 2007). Average annual sunlight, average daily solar radiation at place of birth, and average sunlight intensity are inversely correlated with prevalence of MS and is attributed to the photochemical production of vitamin D from 7-dehydrocholesterol during exposure to ultraviolet-B light (van der Mei et al., 2007; Dickinson et al., 2009; Tremlett et al., 2008).

Since MS has a male-to-female ratio of 1:2, gender obviously has a contribution to susceptibility to developing MS. Women are more likely to develop MS during puberty suggesting that sex hormones are involved with either contributing to, or providing protection against susceptibility to MS (Cardona-Gomez et al., 2001). Also, hormonal fluctuations during menstrual cycles or during postpartum periods are linked to acute relapses and neuroprotection respectively (Zorgdrager and De Keyser, 2002; Langer-Gould et al., 2010). Although women are at a higher risk for developing MS, the disease progression and disability are similar between genders (Tremlett et al., 2006). Also, the age at which MS is acquired affects the disease severity. A younger age of MS onset is associated with a milder disease course; whereas, onset at an older age results in a more severe prognosis (Noseworthy et al., 1983; Simone et al., 2002; Trojano et al., 2002).

Multiple sclerosis is, like many other autoimmune diseases, is T-cell mediated with complex immunologic interactions between antigen and immune components

(McFarland and Martin, 2007). The autoimmune response is directed towards the myelin within the CNS which results in lesions that are profound enough to be visualized by magnetic resonance imaging (MRI). These lesions develop at disease onset and accumulate over time, but the quantification of white-matter lesions has a low correlation with neurologic disability (Rudick and Trapp, 2009). Often unseen during MRI, a more insidious process occurs that leads to the loss of axons, diffuse damage to white matter, epitope spreading, and progressive loss of gray matter. It is the loss of the gray matter that correlates well with cognitive and physical disability (Fisniku et al., 2008). Autoimmune injury to the gray matter cannot be detected with the use of standard MRI, although brain atrophy can be detected with these techniques. MS patients show an accelerated rate of gray matter atrophy that can be up to 14 times faster than those without MS (Fisher et al., 2008). Non-invasive technologies such as magnetic resonance spectroscopy (MRS) or magnetization transfer imaging (MTI) have revealed biomarkers such as N-acetyl aspartate (NAA). Lower levels of NAA, which exists mostly in neurons, indicate pathology and can contribute to early diagnosis, even at early stages of the disease (De Stefano et al., 2002). These techniques show that abnormalities in NAA levels occur within diffuse neuronal regions and are occurring separately from the pathological regions of focal immune attack (Pascual et al., 2007). Other choline containing compounds are also being explored as possible clinical tools for early diagnosis of MS (Kirov et al., 2010; Kirov et al., 2009).

Diagnosis of MS

As there is no single clinical feature or test sufficient to diagnose MS, criteria have included a combination of both clinical and paraclinical testing. Clinical manifestations involve motor, sensory, and autonomic components, which encompass a vast array of heterogeneity among individuals with MS (Polman et al., 2005). Very few of the clinical symptoms are specific to MS, which makes for difficult diagnosis, although some symptoms are quite common among all people with MS. Lhermitte's symptom, which is the electrical sensation that courses down the spinal cord or limbs, and the Uhthoff phenomenon, where symptoms and fatigue become increasingly disabling with an increase in core body temperature (Polman et al., 2005). Early diagnosis of MS allows for early treatment and management of the disease before CNS injury has compromised the ability to undertake activities of daily living (McDonald et al., 2001). Although MRI shows focal or confluent abnormalities in over 95% of patients displaying early signs of MS, their presence alone cannot complete diagnosis of disease as lesions can appear in people without symptomatic signs of disease as many otherwise healthy older individuals have white matter cerebral lesions (Polman et al., 2005). In contrast to the rather ubiquitous white matter abnormalities found in the brain, white matter lesions detected in the spinal cord are absolutely abnormal at any age (McDonald et al., 2001). The International Panel on MS Diagnosis (McDonald et al., 2001) suggests criteria for diagnosing MS using MRI adopted from studies by Barkhof et al (Barkhof et al., 1997) and Tintore´ et al (Tintore et al., 2000). This requires evidence of at least three of four of the following types of lesions: 1) one gadolinium-enhancing lesion or nine T2 hyperintense lesions if gadolinium-enhancing lesions are not present; 2) at least one

infratentorial lesion; 3) at least one juxtacortical lesion involving subcortical fibers; 4) at least three periventricular lesions. These objective data of dissemination on time and space of lesions is essential for diagnosis, and is supplemented by further investigations such as analysis of cerebrospinal fluid (CSF) and visual evoked potential (VEP). These latter two forms of diagnostics are often used when clinical presentation alone does not allow a diagnosis to be made. CSF analysis can help to provide evidence of a humoral immune response in the CNS by examining the presence of oligoclonal IgG bands using electrophoretic technique (Andersson et al., 1994). VEP can be used to supplement information provided by clinical examination, where most people with MS will have a delayed VEP (Plant et al., 1992)

Courses of MS

Up to 90% of people with MS initially present with symptoms of clinically isolated syndrome (CIS) (Pestalozza et al., 2005). CIS is an acute or subacute neurologic episode of inflammation and demyelination which is not necessarily accompanied by other symptomology and is often associated with silent lesions. CIS may appear in a variety of ways, depending on the region of inflammation/demyelination, such as optic neuritis, spinal cord syndrome, or brainstem dysfunction (Brex et al., 2002; Confavreux and Vukusic, 2006; Confavreux et al., 2000). Individuals that present with CIS that is representative of MS usually develop relapsing remitting (RR) MS and then later secondary progressive (SP) MS (Brex et al., 2002; Frohman, 2003; Miller, 2004). People presenting with CIS have a greater chance of clinically definite (CD) MS if they are under the age of 30, have a history of steroid treatment at the onset of symptoms, the

presence of high activity monofocal lesions on MRI, or the presence of oligoclonal bands

from CSF (Miller, 2004). The total lesion load viewed by T2-weighted MRI is also an

extremely strong predictor of development of CD MS, and up to 80% of patients are

diagnosed of prior clinically silent disease activity by this method. Also, two or more

lesions appearing on an MRI during CIS provide an 85% rate of future relapse (Comi et

al., 2001). Following a CIS event, the diagnosis of CD MS must include at least two

neurological events punctuated by areas of demyelination that occur in separate locations

and at different periods of time within the CNS (Jacobs et al., 2000).

The four main classifications of MS disease course are relapsing remitting (RR),

secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR)

MS. They are based according to the frequency and severity of exacerbations, the ability

to recover from exacerbations, and the cumulative damage caused by these exacerbations.

People that have MS may experience several of these disease courses in their lifetime,

and it is not clear as to which factors may be responsible for differing disease courses

(Sospedra and Martin, 2005). Most individuals, approximately 85% who are diagnosed

with MS, initially have RR MS (Kantarci and Weinshenker, 2005). This disease course is

punctuated by attacks of declining neurologic function termed 'relapses' or

'exacerbations'. These relapses are followed by partial or complete recovery periods, or

remission periods, where no disease progression occurs. Primary progressive MS is a

disease course characterized by increasing neurological dysfunction with no significant

relapsing or remitting phases and occurs in only 10-15% of people who are diagnosed

with MS. The rate of progression varies between individuals and may include temporary

improvements in neurological function and plateaus in progression of disease (Stevenson

et al., 1999). People with PP MS are usually male and are generally older than the average person with MS at initial diagnosis. Diffuse axonal loss and microglial activation are seen in the white matter, in addition to cortical demyelination, atrophy, and intrinsic abnormalities in the grey and white matter. Conventional immunomodulatory therapies, such as interferon beta and glatiramer acetate appear to be ineffective (Miller and Leary, 2007). The third MS classification is SP MS. This disease course is initiated by a period of RR MS that eventually develops into a more chronic, progressive type of disease course. As many as ~50% of people with RR MS will develop a SP disease course after 10 years and approximately 90% will develop SP MS after 25 years (Kantarci and Weinshenker, 2005). Clinical exacerbations usually occur during the transition period from RR MS to SP MS and most will eventually develop a steady decline of neurologic function over a period of months to years (Stevenson et al., 1999). Recovery from relapses is related to the ability of the CNS to adapt and recruit adjacent neuronal structures to restore neurological function. Despite the plasticity of the CNS, the amount of relapses has a finite capacity to restore function, as repeated relapses increase neuronal damage and lead to accumulated disability (Zaffaroni, 2005). The last type of disease course classification is PR MS. This is a relatively rare form of disease course that is seen in approximately 5% of the MS population. They experience a chronic increase in disease severity from the beginning that includes periods of exacerbations with little or no recovery following the relapse (Lublin and Reingold, 1996). MS affects each individual differently, so initial classification may be difficult for physicians.

MS Symptoms

The symptoms associated with MS are extremely variable and reflect the regions of the CNS that have been affected. Symptoms often appear in a subtle, subacute fashion that last for days to weeks, or acutely, such as loss of vision (optic neuritis) occurring in many people with MS early in the disease course. Optic neuritis usually presents with painful monocular vision loss in younger patients with spontaneous improvement in vision occurring over the course of several weeks. Most acute symptoms last an average of 6-12 weeks with 90% recovering almost completely within this time frame (Clark et al., 2010). The typical symptoms experienced by people with MS are fatigue, depression, heat sensitivity, cognitive dysfunction, sensory perturbations, gait disturbances, ataxia, vertigo, pain, bowel and bladder dysfunction, muscle spasticity, and other symptoms that are variable between individuals. The more common and chronic of these symptoms is fatigue, Uhthoff's phenomenon, cognitive decline, bladder and bowel dysfunction (Kantarci and Weinshenker, 2005). Some of these symptoms often appear weeks to months before the first exacerbation, and can be brought about by an increase in body temperature upon exposure to hot weather or exercise. It has been proposed that Uhthoff's phenomenon occurs due to a decrease in axonal conduction in partially demyelinated fibers when body temperature is increased (Rudick and Trapp, 2009). Other initial symptoms of MS include trigeminal neuralgia, tonic spasticity, and facial myokymia. Most of these MS symptoms are episodic and are commonly followed by a progressive decline in function with gradually increasing disability (Compston and Coles, 2002). Clinical manifestations and long term neurologic damage may be mild to severe between different individuals. Despite the wide variability between individuals, typical

disease course involves cumulative and progressive disability with time. In most people with MS, the mean duration of symptoms is 15 years with approximately 30% of all people with MS eventually requiring the use of a wheelchair (Richards et al., 2002). Eventually after 15 years, 50% of people with MS progress to a Kurtzke Expanded Disability Status Scale (EDSS) score of 6 or greater. People who achieve an EDSS score of 8.5 or greater are commonly unable to move their arms or legs (Weinshenker, 1996).

Types of Lesions

Several types of demyelinating lesions have been characterized in the brains of humans with multiple sclerosis during postmortem analysis (Peterson et al., 2001). Pattern one and two lesions involve both gray and white matter and with infiltration of T-cells and macrophages entering the cortical CNS vasculature (Derfuss et al., 2009). The blood-brain barrier (BBB) is activated, or inflamed, possibly by these T-cells which permit circulating anti-myelin glycoprotein antibodies to gain access to perivascular areas (Zivadinov and Cox, 2007). Pattern two lesions are similar to pattern one type lesions, yet there is a prominent humoral component consisting of activation of compliment by anti-myelin glycoprotein antibodies. Pattern one and two lesions show promise in having the potential for remyelination (Zivadinov and Bakshi, 2004). In pattern three lesions, reactive T-cells within lymphoid follicles in the subarachnoid space mediate the activation, thus permitting anti-myelin Ab to gain access from the subarachnoid space (Derfuss et al., 2009). There is severe loss of myelin associated glycoproteins and oligodendrocytes undergo apoptosis (Qin et al., 2010). MRI reveals that acute and chronic lesions exist, along with a varied array of lesion types in individuals with MS,

demonstrating a dynamic pattern of disease. As individuals with MS age, their lesions undergo less inflammation during relapse yet there is an increase in axon loss and glial scarring (Brex et al., 2002). Due to this wide variation in pathology within individuals, some researchers suggest that distinct pathological subtypes of MS exist and that it is a heterogeneous disease.

Treatments for MS

Some of the most common forms of treatment for RR MS have been interferon beta (IFN-β) and glatiramer acetate (GA) (Paty et al., 2001; Johnson et al., 1995). These drugs help to shift the immune response away from increased inflammation, although they do have side effects and only moderately reduce the rate of relapses (Martin, 2010). More aggressive treatments include a monoclonal antibody type of drug called Natalazumab which acts by interfering with the migration of T-cells into the CNS (Miller et al., 2003). It is more effective than IFN-β and GA, but greater than 30 individuals receiving the treatment developed an often fatal brain infection called progressive multifocal leukoencephalitis (Tourbah, 2008). Mitoxantrone is a drug used to treat RR MS and early phase SP MS. This drug has shown promise in augmenting the progression of MS, although heart toxicity has been reported as well as the development of secondary leukemias in 2.8% of individuals receiving the treatment (Pascual et al., 2009). All of these aforementioned treatments require frequent injections or intravenous infusions. An oral alternative to these medications comes in the form of an immunomodulatory agent called fingolimod, or FTY720. This drug appeared to be well tolerated and successful in two large clinical trials, the FREEDOMS trial (Kappos et al., 2010), and the

TRANFORMS trial (Cohen et al., 2010). FTY720 is a sphingosine-1-phosphate (S1P)

receptor agonist that inhibits T-cell migration from the immune organs to the periphery

and then into the CNS (Sospedra and Martin, 2005; Bielekova et al., 2000; Yednock et

al., 1992). This drug is similar to the lipid sphingosine-1-phosphate which acts to

promote lymphocyte homing in the immune organs such as the thymus and lymph nodes

(Chiba et al., 1998; Mandala et al., 2002). Subsequently, the effector T-cells remain in the

lymph nodes and their population within the circulation remains reduced. Yet another

highlight of FTY720 is that it can readily cross the BBB and interact with the S1P

receptors on astrocytes and oligodendrocytes, which then provides an environment for the

remyelination process (Miron et al., 2008). Although this drug has proven to be more

effective than interferon beta treatment, there have been moderately serious herpesvirus

infections, cardiovascular complications, macular oedema, benign and cancerous tumors,

hemorrhagic encephalitis, and other infections (Cohen et al., 2010; Leypoldt et al., 2009).

With the vast amount of side effects experience by people with MS when taking these

drugs, other treatments or interventions are desirable. Exercise has the potential to

modulate the immune system and provide endogenous mechanisms that lead to the

maintenance of cell populations in the CNS. Some of these mechanisms are conferred by

a family of signaling molecule called neurotrophins and their ligation with signal

transducing receptors.

Brain Derived Neurotrophic Factor

Neurotrophins are a small family of signaling proteins that regulate the survival,

death, and differentiation of neurons during embryonic development as well as

homeostasis and repair throughout life (Lewin and Barde, 1996). They include brain

derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3),

neurotrophin 4/5 (NT-4/5) and some related ligands. Pro-neurotrophins are cleaved by

metalloproteases after synthesis to form a mature neurotrophin protein that is 13-15 kDa

in size (Roux and Barker, 2002). These neurotrophins bind to a common family of

tropomyosin related kinase (Trk) cell surface receptors with high affinity and are

produced by a myriad of cells in the body including the nervous system, immune system,

endothelia, smooth muscle and skeletal muscle (Nockher and Renz, 2003).

Brain-derived neurotrophic factor (BDNF) is a growth factor which modulates

function, survival, proliferation, differentiation, myelination, apoptosis, and axonal

growth in the central nervous system (Arevalo and Wu, 2006). It is produced by a myriad

of different cells within the CNS, and is also thought to be a possible molecular effector

of immune responses in the CNS and peripheral immune system (Hohlfeld et al., 2006) as

it is synthesized and released by activated immune cells (De Santi et al., 2009b). BDNF is

found in higher concentrations within MS lesions and has been shown to prevent axonal

and neuronal damage after pathological insults. BDNF has a short serum half-life and

does not readily cross the blood-brain barrier (BBB). Makar et al. found that genetically

engineered bone-marrow stem cells (BMSC) that can cross the BBB and deliver BDNF

to the CNS can reduce inflammation and apoptosis in SJL/J mice induced with EAE. The

reduction in apoptosis seen in the brain and spinal cord was mediated by activation of

pathways that lead to an increase in Akt and Bcl-2 levels that are consistent with the

proposed mechanism by which BDNF exerts its anti-apoptotic effects. The pro-apoptotic

cathepsin-B, a protease found to be increased in inflammatory lesions in EAE and MS,

was also found to be reduced with BDNF treatment (Makar et al., 2008). The BMSC BDNF delivery method also reduces demyelination in the EAE model and increases remyelination through activation of post-mitotic oligodendrocytes and oligodendrocyte progenitors (Makar et al., 2009). In experimental axotomy, BDNF can rescue and promote the survival of neurons (Gravel et al., 1997) and increase oligodendrocyte proliferation and axonal remyelination (McTigue et al., 1998).

BDNF has also been found to be produced by B-cells, platelets, macrophages, endothelia, smooth muscle and skeletal muscle cells, microglia, CD4+ T-cells (both Th1+ and Th2+) and CD8+ T-cells (Nockher and Renz, 2003; Edling et al., 2004; De Santi et al., 2009a). BDNF is a crucial protein in the maturation of B-cells and is produced within hematopoietic bone marrow. In BDNF deficient mice, the number of B-cells in the spleen is reduced and B-cells become arrested in the pre-BII phase (Schuhmann et al., 2005). BDNF is also expressed in the stroma of the thymus and acts as a growth factor for T-cell precursors. The BDNF receptor, TrkB, is also present in these CD4+ CD8+ immature thymocytes, which is suggestive of an interaction between BDNF from the stroma and immature T-cells within the thymus (Maroder et al., 1996).

Weinstock-Guttman et al. used non-conventional MRI measures such as diffusion weighted imaging and magnetization transfer imaging to show a correlation between immune cell BDNF secretion and increased inflammation in the white matter (WM) of people with MS. They also found that the maintenance of white matter volume (WMV) was associated with higher secretion of BDNF by peripheral blood mononuclear cells (PBMCs) (Weinstock-Guttman et al., 2007). Lymphocytes from RR MS subjects secreted higher levels of BDNF during an exacerbation and recovery compared with a stable

remission period, indicating that BDNF is upregulated during periods of increased

disease activity (Sarchielli et al., 2002). This is consistent with the increased need for

CNS neuroprotection during inflammation as seen with the number of contrast enhancing

lesions, which are known to be associated with increases in brain inflammation. The

lowest levels of BDNF secretion from PBMCs was seen with a group of people with SP

MS who had increased CNS deterioration and disability (Sarchielli et al., 2002). There is

a decrease in BDNF secretion from PBMCs with increased disease duration, which

suggests that immune cell secretion of BDNF is involved with neuroprotection earlier in

the MS disease course (Weinstock-Guttman et al., 2007).

In autopsy brain tissue from people with MS, Stadelmann et al. found that lesions

with ongoing demyelination show higher ratios of BDNF positive immune cells. These

BDNF producing cells found in and around the lesions are mainly macrophages,

microglia, and T-cells. The BDNF positive cells were found to be present in early stage

lesion formation, whereas in chronic inactive lesions, only a few BDNF producing cells

were found. This observation lends support to the idea that inflammatory cells provide

neurotrophic support, as the neurons in the immediate vicinity of inflammation have

increased expression of TrkB receptors. This neurotrophin/receptor interaction seems to

be a major factor that helps to preserve neurons in a microenvironment of neurotoxicity

(Stadelmann et al., 2002).

BDNF also has some immunomodulatory effects by downregulating MHC class II

molecules in microglia, which are responsible for antigen presentation to CD4+ T-cells

{{307 Neumann,H. 1998; }}. CD4+ T-cells are known to be modulators of the

autoimmune response to myelin antigens in EAE and MS (Sospedra and Martin, 2005). A

shift to a Th2 T-cell phenotype milieu of cytokines induces the upregulation of BDNF in glial cell cultures, while a Th1 cytokine cocktail downregulates the genes that both produce neurotrophins and their receptors (Lisak et al., 2009). Current immunomodulating therapies such as IFN-β and GA indirectly stimulate the expression of BDNF, as well as other neurotrophin genes in glial cells and modulate the production of a Th2 cytokine pattern (Lisak et al., 2009).

Tropomyosin Related Kinase-B Receptors

Tropomyosin related kinase receptors were discovered and named after an oncogene that consists of the first seven of eight exons that transcribe non-muscle tropomyosin. Mature BDNF binds two different types of tropomyosin-related kinase (Trk) transmembrane spanning receptors. This includes the full length signal transducing isoform high affinity receptor (gp145trkB) and the truncated gp95trkB isoform which lacks the cytoplasmic catalytic kinase domain (Klein et al., 1990). BDNF also weakly binds the p75^{NTR} low affinity neurotrophin receptor and is required for high affinity binding to the Trk receptors (Volosin et al., 2006). The gp145trkB is most commonly expressed on neurons and its signal transduction promotes most of the known biological effects of BDNF. These receptors are found in high concentrations surrounding the areas around lesions rich in BDNF producing T-cells in autopsy brains from people with MS, suggesting that there are immune-mediated neuroprotective mechanisms involved in MS lesions (Stadelmann et al., 2002).

Each full-length Trk receptor contains a single transmembrane domain and a single cytoplasmic tyrosine kinase domain. The most distal extracellular portion of Trk

receptors contain an array of three leucine rich motifs flanked by two cysteine clusters. Two immunoglobulin-like C2 type domains (Ig-C2) are arranged more proximal to the membrane and adjacent to a single transmembrane domain. The intracellular cytoplasmic domain contains three tyrosine kinase domains in addition to tyrosine-containing motifs. Phosphorylation of the tyrosine domains regulates tyrosine kinase activity and allows for phosphorylation dependent recruitment of adapter proteins that lead to intracellular signaling cascades (Kaplan and Miller, 2000; Huang and Reichardt, 2003).

Ligand binding occurs at the Ig-C2 domain at several possible locations proximal to the membrane and leads to dimerization of Trk receptors and autophosphorylation of the intracellular tyrosine domains. Point mutations at the Ig-C2 region can lead to spontaneous dimerization and subsequent activation of kinase activity (Arevalo and Wu, 2006). Phosphorylation of the tyrosine domains creates docking sites for adapter protein which activate several signaling cascades including the phosphatidylinositide 3'-kinase (PI3K)/protein kinase B (Akt), phospholipase C (PLCγ)/phosphokinase C (PKC), and the Ras-MAPK/Erk signaling cascades that lead to the activation of transcription factors that are involved in cell survival, growth, and a positive feedback increase in transcription of BDNF (Tao et al., 1998).

The phosphorylated tyrosines of Trk receptors have been the focus of much research. Phosphotyrosine-490 (Y490) with the adapter proteins Shc, Frs2 and IRS1/2 provide for the activation of Ras and PI3K and lead to cell signaling through the Ras-MAPK/Erk and PI3K/Akt signaling cascades respectively. Activation of the TrkB receptors at the Y490 domain prevents the loss of populations of neurons (Jang et al., 2010) and deletion of Shc family adapters leads to a significant loss of neurons within the

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superior cervical ganglia showing that phosphorylation at Y490 leads to the recruitment

of Shc adapter proteins and the activation of pathways that regulate cell death (Sakai et

al., 2000). Additional adaptors also play a role in pathway activation after Y490

phosphorylation. These include the tyrosine phosphorylation on the adapter proteins

rAPS and SH2-B, which associate with Grb2, SOS and Ras, leading to phosphorylation

and activation of PI3K (Huang and Reichardt, 2003). The phosphorylation of

phosphoinositides such as PI3 by PI3-kinase leads to the phosphorylation and activation

of the serine-threonine kinase Akt (a.k.a. PKB or pAkt), which can then activate several

proteins that regulate cell survival and growth.

Apoptosis

Programmed cell death resulting in apoptosis is an evolutionary conserved and

genetically regulated process that is critical for normal development. Any defects in the

cell death machinery may contribute to pathologies ranging from oncogenesis to

autoimmunity to neurodegeneration (Putcha et al., 2003). In mammalian cells, apoptosis

can be divided into two broad categories. These include the intrinsic and extrinsic

pathways that often converge on each other at certain points in the signaling cascade. The

intrinsic pathway requires the release of mitochondrial proteins such as cytochrome-c, a

heme protein associated with the mitochondrial membrane, and Smac/DIABLO, an

inhibitor of apoptosis protein (IAP)-binding protein, to activate the cleavage of caspases.

Once released from the mitochondria, cytochrome-c promotes the assembly of the

apoptosome, which results in caspase-9 activation and the propagation of a caspase

cascade. Extrinsic pathways activate the cleavage of caspases more directly but are enhanced by the mitochondrial pathway.

The activation of the Ras-MAPK/Erk and PI3K/Akt pathways can inhibit activation of apoptosis by phosphorylation of the pro-apoptotic proteins and lead to increased expression of anti-apoptotic proteins in the mitochondrial membrane which ultimately inhibit activation of caspases (Huang and Reichardt, 2003). Bax and Bad, which are Bcl-2 family proteins, are recruited to the mitochondrial membrane surface and initiate apoptosis through sequestration of Bcl-XL (B-cell lymphoma extra-large), another Bcl-2 family protein, to promote the formation of multimeric pore complex on the mitochondrial membrane and results in the release of cytochrome-c and other pro-apoptotic factors (such as Smac/DIABLO) from the mitochondria (Adrain et al., 2001). This mitochondrial outer membrane permeabilization leads to formation of the apoptosome and activation of caspases. BAD is directly phosphorylated by pAkt and can then bind with 14-3-3 proteins to prevent the interaction of BAD with Bcl-2 and Bcl-XL, which allow for inhibition of the proapoptotic protein Bax (Datta et al., 1997). Activated Akt also phosphorylates proapoptotic proteins such as glycogen synthase 3-β (GSK3β) and the inhibitor of NFκB, IκB, which results in liberation of NFκB and subsequent transcription of genes that lead to neuronal survival (Foehr et al., 2000).

Neurotrophin deprivation has been shown to activate the c-Jun N-terminal kinases (JNKs) that culminate in activation of the BCL-2 proteins (BIM and HRK) and the ultimate release of BAX-dependent cytochrome-c release, caspase cleavage, and apoptosis (Putcha et al., 2003). Growth factors such as NGF and BDNF can promote cell survival by activating their respective Trk receptor and tyrosine kinase signaling through

the PI3K and its downstream target, Akt. After activation, pAkt phosphorylates the BCL-2 family member, BAD, which allows BAD to be sequestered by the 14-3-3 protein and inhibits the cascade of events that lead to mitochondrial membrane permeability and ultimately, apoptosis (Datta et al., 1997). Akt also phosphorylates the caspase proteases and inhibits caspase cleavage (Cardone et al., 1998). The PI3K/Akt pathway also regulates apoptosis by suppressing the expression of death genes by phosphorylating the Forkhead box family of transcription factors (Biggs et al., 1999). Nguyen and colleagues found that both NGF and BDNF prevent staurosporine induced apoptotic activity and caspase cleavage by upregulation of the phosphorylation of the Trk receptor (TrkA and TrkB) in hippocampal H19-7 cells. Inhibition of the Trk receptor by K252a abolished the protective effect of both NGF and BDNF. Impairment of the PI3K/Akt pathway by overexpression of a dominant negative Akt phenotype also abolished the protective effects of NGF and BDNF, and siRNA knockdown of Akt was also able to block the pro-survival effects of the neurotrophins (Nguyen et al., 2010). These data show that activation of the TrkA and TrkB receptors and subsequent activation of the PI3K/Akt pathway confer an anti-apoptotic effect on staurosporine-induced neurotoxicity. Since TrkB receptors are widely distributed throughout the CNS and TrkA receptors are reported to be sparse in the CNS, especially in the hippocampal and cortical neurons (Friedman, 2000), then BDNF upregulation and activation of the TrkB receptor should confer neuroprotection in neurological diseases that cause widespread insult in the CNS such as MS.

Hobom et al. examines retinal ganglion cells in the EAE models and found that apoptosis started to occur prior to the onset of clinical symptoms. TUNEL positive cells

were detected seven days before clinical disease onset with the highest number of apoptotic cells detected on day one of clinical disease symptoms. At day eight after EAE disease, apoptotic cells were still detected, yet at a lower cell count. Caspase-3 was found to be activated at the same time points as TUNEL detection of apoptotic cells along with a reduction in p-Akt. Bcl-2 and Bax also follow the same timecourse, with decreases in Bcl-2 and increases in Bax (Hobom et al., 2004). This is important from a study design perspective, where attempting to detect apoptotic cells at a later timepoint in the disease course may lead to a null finding.

Li and colleagues used APP/PS1 mice, which is a model of familial Alzheimer's disease, to measure the ratio of phosphorylated TrkB (Thr 515) receptors to Trk receptors in the hippocampus and found that this ratio is decreased in APP/PS1 mice. They also measured downstream signaling molecules that result from phosphorylation of TrkB receptors by BDNF. In the APP/PSI mice, pAKt (Ser 473) to Akt and pERK to ERK were significantly decreased when compared to wild type mice. Active caspase-3 fragment to caspase-3 was increased while the Bcl-2 to Bax ratio was decreased in the Alzheimer's model. They also noted that neural apoptosis was increased by ~40% in the APP/PS1 mice after Nissl body staining. Their data suggests that decreased phosphorylation of the TrkB receptors leads to decreased activation of pathways that provide neuroprotection and subsequent increases in apoptosis are witnessed (Li and Liu, 2010).

Das et al. examined the time course of caspases and intermediates of apoptotic pathways in EAE animals and found that proteins involved in apoptosis were most profound on days 8-10 following induction of EAE. TUNEL labeling revealed that

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neuron death was most substantial at day 11 after EAE challenge and was a result of the increases in pro-apoptotic products. They found that phosphorylated BAD was decreased in the EAE model and that was correlated with increases in calpain, which activates the 60kDa calcineurin, which dephosphorylates BAD. Caspase 8, 9, 12, and 3 active fragments were also found to be increased maximally on days 8-10, just before the greatest increase in apoptotic cells. Increase in Bax to Bcl-2 ratio during EAE onset show that the mitochondrial pathway of apoptosis was involved (Das et al., 2008).

Effects of Exercise

Treatments or interventions that can lead to increases in TrkB signaling are beneficial in terms of maintaining myelination and inhibiting apoptosis. Exercise appears to be a promising intervention that may lead to neuroprotection in people with MS and the EAE animal model (Castellano and White, 2008a; Rossi et al., 2009). In other neurodegenerative diseases the effects of exercise have only been moderately described. This previous research has shown that exercise delays the motor deficits in animal models of stroke (Ding et al., 2005; Li et al., 2004), amyotrophic lateral sclerosis (Kirkinezos et al., 2003), Huntington's disease (Pang et al., 2006; van Dellen et al., 2008), progressive motor neuronopathy (Ferrer-Alcon et al., 2008), spinal muscular atrophy (Grondard et al., 2005), Parkinson's disease (Mabandla et al., 2004; O'Dell et al., 2007), and EAE animal models (Rossi et al., 2009; Le Page et al., 1996). Little is known about the mechanisms behind the attenuation of neurodegeneration from exercise. Rossi et al. found that voluntary wheel running in C57BL/6 mice restored the sensitivity of striatal GABAergic synapses to the stimulation of cannabinoid CB1 receptors (Rossi et

al., 2006). They also found that striatal dendritic spine loss was decreased in an exercise group when compared to controls. Exercise may act by modulating the activity of striatal neurons through the activation of dopamine (DA) signaling and upregulation of FosB in this brain region (Werme et al., 2002). The striatum receives a large amount of DA innervation from the substantia nigra which increases DA release in the striatum during voluntary exercise and may regulate signaling pathways that lead to neuroprotection (El Rawas et al., 2009).

Exercise promotes recovery in the CNS after traumatic injury (Jones et al., 1999) and enhances neurogenesis (Gomez-Pinilla et al., 2002). Exercise also increases the expression of BDNF and NT-3 in the spinal cord and skeletal muscle after voluntary exercise (Gomez-Pinilla et al., 2001; Ying et al., 2003) and upregulates the capacity for axonal outgrowth from cultured neurons *in vivo* (Molteni et al., 2004). The role of exercise is important in plasticity of the neuromuscular system and is a means for the increased endogenous production of neurotrophins that may impact neuromuscular function and neuroprotection from autoimmune insults in EAE and MS.

BDNF and exercise

BDNF is encoded by a gene that is regulated by activity-dependent cAMP-response element binding protein (CREB) family of transcription factors that are initiated by increased Ca^{+2} that is upregulated through the activation of NMDA receptors and/or voltage gated Ca^{+2} channels (Tao et al., 1998; Shieh et al., 1998). Exercise induced BDNF increases are suppressed in mice that are lacking the NMDA receptor epsilon-1 subunit (Kitamura et al., 2003). BDNF not only leads to cell survival and proliferation,

but can contribute to regulation of long-term potentiation by enhancing glutamatergic synaptic transmission in hippocampal neurons (Li et al., 1998). Berchtold et al. found that BDNF is increased in the hippocampus immediately after exercise and remains elevated for two weeks after exercise cessation. The BDNF levels showed a positive correlation with cognitive accomplishment in radial water maze memory performance, which was attenuated when anti-TrkB antibodies were administered into the hippocampus (Berchtold et al., 2010).

Choi et al found that an approximately 50% reduction in BDNF through selective gene ablation in hippocampal neurons impairs the survival of progenitor cells and impairs dendritic development (Choi et al., 2009). These deficits are only moderately restored by exercise in the conditional knock-out mice, suggesting that BDNF contributes largely to cell survival, proliferation, and dendritic development in the hippocampus.

TrkB signaling and exercise

The level of phosphorylation of the kinase domains of the TrkB receptor is highly correlated with the availability of BDNF. Phosphorylated TrkB receptors are increased in various regions of the CNS after four weeks of exercise (Liu et al., 2008a) and ablation of TrkB expressed in neural progenitor cells using hGFAP-Cre can inhibit exercise-induced increases in hippocampal cell proliferation and neurogenesis (Li et al., 2008).

Some researchers have shown some evidence that exercise does not alter immune cell infiltration into the CNS during EAE, therefore the neuroprotection conferred by exercise is not by a reduction in immune response, but more likely due to intrinsic mechanisms (Rossi et al., 2009). Macias et al. found that TrkB mRNA was increased in

the spinal cord ventral horn neurons that consisted of precursor and mature oligodendrocytes in Wistar rats after 28 days of treadmill exercise. TrkB mRNA transcripts were also found throughout the dorsal horn, with a particularly high density in the superficial laminae (Macias et al., 2007). Other investigators have also confirmed the increases in TrkB receptors in the grey matter of the spinal cord, with the majority of the TrkB positive cells being oligodendrocytes. Skup et al. demonstrated that treadmill walking one kilometer per day for four weeks lead to increased gp145TrkB small cells of the spinal gray matter with no increases in gp95TrkB in the lumbar spinal cord of adult Wistar rats (Skup et al., 2002). Wu and colleagues used an animal model of Parkinson's disease and found that exercise protected dopaminergic neurons from lipopolysaccharide (LPS) degeneration. Inhibiting the TrkB receptor via intracerebroventricular injection of K252a abolished the exercise induced protection of dopaminergic neurons from LPS injury. Intracerebroventricular injection of BDNF alone was sufficient to protect from LPS induced injury suggesting that exercise mediates its neuroprotective effects through BDNF/TrkB signaling cascades (Wu et al., 2011). These observations bring forth the idea that CNS cells become more responsive to neurotrophic stimuli and more resistant to apoptosis after exercise.

There are only a few studies examining the effects of exercise on mechanisms of neuroprotection in the EAE animal model. Rossi et al showed that exercise rescued the function of the endocannabinoid system, prevented dendritic spine loss in the striatum, and reduced overall clinical disease severity after induction of EAE (Rossi et al., 2009). Exercise can also delay the onset of clinical signs of disease in adoptive transfer of EAE in mice forced to run on treadmills (Le Page et al., 1996). EAE mice that have chronic

exposure to running wheels show delayed onset of disease and decreased disease severity as well as an increase in BrdU positive subventricular cell proliferation as compared to EAE animals without running wheels. The exercising EAE mice have increased cell proliferation that favors oligodendrocyte commitment into demyelinated lesions (Magalon et al., 2007). To date, no researchers have examined the ability of exercise to influence the regulation of TrkB receptors in the CNS of the EAE animal model.

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CHAPTER 3

EXERCISE CONFERS NEUROPROTECTION IN EXPERIMENTAL

AUTOIMMUNE ENCEPHALOMYELITIS[1]

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Abstract

Multiple Sclerosis (MS) is an autoimmune disease that affects the central nervous system resulting in accumulated loss of cognitive, sensory, and motor function. In this study we aimed to evaluate the neuropathological effects of voluntary exercise in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Two groups of C57BL/6J mice were injected with an emulsion containing myelin oligodendrocyte glycoprotein (MOG) and then randomized to housing with a running wheel or a locked wheel. Exercising EAE mice exhibited less severe neurological disease score and later onset of disease when compared to sedentary EAE animals. Immune cell infiltration and demyelination in the ventral white matter tracts of the lumbar spinal cord were significantly reduced in the EAE exercise group compared to sedentary EAE animals. Neurofilament immunolabeling in the ventral pyramidal and extrapyramidal motor tracts displayed a more random distribution of axons and apparent loss of smaller diameter axons with a greater loss of fluorescence immunolabeling in the sedentary EAE animals. In lamina IX grey matter regions of the lumbar spinal cord, sedentary animals with EAE displayed a greater loss of α-motor neurons when compared to EAE animals exposed to exercise. Our findings provide evidence that voluntary exercise results in reduced and attenuated disability, reductions in autoimmune cell infiltration, and preservation of axons and motor neurons in the lumbar spinal cord of mice with EAE.

Keywords: Multiple sclerosis, experimental autoimmune encephalomyelitis, neuroprotection, exercise, neuropathology, spinal cord.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) and is the leading cause of neurologic disability in young adults (Ramagopalan et al., 2010; Richards et al., 2002). MS causes impaired mobility that inhibits or discourages activities of daily living (White et al., 2006; Slawta et al., 2003). In people with MS, infiltrating lymphocytes and macrophages along with activated astrocytes and microglia lead to the hallmark signs of demyelination and axonal transection that occur in white matter tracts of the CNS (Lassmann, 2007). In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), similar patterns of infiltration of a heterogenous population of myelin-reactive inflammatory cells occur (Jager et al., 2009). Differences in MS and EAE are based upon the location of infiltrating immune cells. In MS, infiltration is typically initiated in the brain and affects the brainstem and spinal cord later in the course of the disease. Conversely, EAE typically begins in the spinal cord and leads to initial motor impairments at disease onset which allows for the clinical assessment of EAE (Brown and Sawchenko, 2007). Although the relationship between these anatomical differences is not well understood, a mechanism has recently been described where upregulation of chemokines in the dorsal blood vessel at the fifth lumbar spinal cord are induced by activation of sensory neurons (Arima et al., 2012). Examining this region where autoreactive cells are gated across the blood-brain barrier (BBB) in a model that is exposed to chronic motor and concomitant sensory activation is crucial for determining the potential efficacy of exercise as a therapeutic candidate for MS.

There is a strong relationship between immune cell infiltration, demyelination, axon loss, and permanent neurological disability in MS and EAE (Trapp et al., 1998; Forte et al., 2007). Axonal loss correlates with neurological impairment in humans (Matthews et al., 1998) and in animal models of MS (Wujek et al., 2002). Axonal loss not only occurs in demyelinating lesions but also in white and grey matter early during the course of EAE (Herrero-Herranz et al., 2008). Although there are many mechanisms by which axon degeneration occurs and progresses (Stys, 2005; Coleman and Perry, 2002; Linker et al., 2005), only a few protective pathways have been described in the EAE model (Forte et al., 2007; Lee et al., 2012; Gold et al., 2007). Therapies that inhibit trafficking of autoreactive cells into the CNS reduce the occurrence of demyelinating lesions in people with MS (Rudick et al., 2009; Goodin et al., 2008; Linker et al., 2005), but do not prevent long-term CNS axon loss (Trapp et al., 1998). This is due, in part, to the sustained glial activation, and show that the chronic activation of mechanisms intrinsic to CNS innate immunity contributes to axonal loss in MS (Choi et al., 2011; Soulika et al., 2009).

Spinal cord autopsy specimens show that there is a 48% loss of α-motor neurons in the lumbar spinal cord of people who had MS when compared to non-MS controls (Vogt et al., 2009). In the EAE animal model, similar levels of α-motor neuron loss have been reported (Vogt et al., 2009; Aharoni et al., 2005). Motor neurons are consistently lost due to apoptosis as disease progresses, but such changes can be attenuated with current MS pharmacological treatments (Aharoni et al., 2005). In spinal muscular atrophy mouse models, exercise has been shown to support the survival of motor neurons in the lumbar spinal cord (Grondard et al., 2005). Some of the beneficial effects of chronic

exercise act directly on the molecular machinery of neural tissue, in which exercise regulates the expression of a broad array of genes involved in pro-survival pathways (Clark et al., 2011).

Exercise can lead to neuroprotection from forebrain ischemia (Stummer et al., 1994), neurotoxin insult, and inherited neurodegeneration affecting the cerebellum (Carro et al., 2001). Exercise has also been shown to preserve or enhance physical function in people with MS, suggesting that exercise may confer neuroprotection (Heesen et al., 2006; Castellano and White, 2008). Some evidence reveals that exercise in EAE animal models may be effective at attenuating disease onset and reducing disease severity (Rossi et al., 2009; Le Page et al., 1996). How exercise works to promote neuroprotection in animal models of disease has been elusive and more emphasis on the topic of exercise as a serious therapeutic treatment is needed. Our aims were to determine if voluntary exercise prior to and during the onset of EAE can lead to changes that maintain motor abilities and ameliorate the neuropathology of the disease.

Experimental Procedures

Experimental Animals

C57BL/6J mice (Jackson Laboratories), 16-20g, 10-weeks of age, were housed singly at a 12:12 hour light/dark cycle in a temperature-controlled environment (22 °C; 50–60% humidity). Standard laboratory irradiated chow (Purina PicoLab Rodent Diet 20) and water was available ad libitum. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

EAE Induction

Ten animals were injected with 200 µg of myelin oligodendrocyte glycoprotein 35-55 (MOG 35-55) (Biomatik) in 200 microliters of complete Freunds adjuvant (CFA) containing 1 mg/mL heat-killed Mycobacterium tuberculosis (MP Biomedical). The CFA was supplemented with mortar-ground, heat-killed Mycobacterium tuberculosis (Difco H37Ra) for a total concentration of 5mg/mL. The MOG/CFA emulsion was subcutaneously injected at two sites; one on the flanks and one behind the neck (100µL each). 400 nanograms (800ng total) of *Bordatella pertussis* toxin was injected intraperitoneally on the MOG/CFA injection day (day 0) and again 24 hours later (day 1).

Disability Scoring and Monitoring

All mice were scored daily for EAE using a standard 6-point disability scale (Beeton et al., 2007). (0 = no disease; 1 = limp tail; 2 = mild paraparesis, ataxia; 3 = moderate paraparesis; 4 = complete hind limb paralysis; 5 = complete hind limb paralysis and incontinence; 6 = moribund, difficulty breathing, does not eat or drink).

Exercise Protocol

Animals were randomly assigned to either a voluntary exercise (n=4) or sedentary group (n=4). Animals in the exercise group were individually housed and had access to a running wheel within their cage 24 hours/day, 7 days/week. The sedentary group was exposed to the same environment, but the running wheels were locked. An electronic system was used to measure running distance, time, and average velocity performed in a 24-hour period.

Histopathology

After 25 days the mice were euthanized with CO_2 and perfused transcardially with PBS followed by 4% paraformaldehyde. Spinal cord sections were postfixed in 4% paraformaldehyde for four hours then 20% sucrose solution for 24 hours. Fixed spinal cords were cut at 20 μm coronal sections on a cryostat at -20 °C. Direct mounted sections were submerged in .05% luxol fast blue (Sigma) solution and incubated for 12 hours at 57 °C. Excess stain was rinsed in 95% EtOH followed by dH_2O. Sections were then briefly differentiated in .05% lithium carbonate solution followed by 70% EtOH until white and grey matter could be distinguished. Sections were counterstained in 0.1% cresyl violet acetate (Sigma) solution, rinsed in dH_2O and dehydrated in graded alcohols. Slides were then cleared in a series of xylene baths before being mounted with a xylene based mounting medium. For lumbar sections used exclusively for measures of demyelination, only the luxol fast blue portion of the staining protocol was performed.

Analysis of Infiltration and Demyelination

Brightfield images of luxol fast blue/cresyl violet sections were photographed using a light microscope (Zeiss) with a digital camera. All images were collected using similar acquisition parameters. Photomicrographs of at least 10 sections per animal, 4 animals per group, were analyzed with ImageJ software (Version 1.44p. NIH). Analysis of cresyl violet and luxol fast blue staining include the following steps: (1) the ventral white matter regions of the lumbar images were sectioned by drawing lines separating the left and right hemispheres and ventral and dorsal sections with the lines intersecting at the central canal, (2) the ventral white matter of the spinal cord was manually outlined,

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segregated, and converted to 8-bit greyscale images, (3) the image was converted to a binary image (4), and then area of cresyl violet or luxol fast blue staining was measured and expressed as a percent of total area.

Immunohistochemistry

Lumbar spinal cords were cut at 20 μm coronal sections on a cryostat at -20 °C. Sections were direct mounted on charged slides and blocked with 10% normal goat serum (NGS) in 0.3% Triton-X in PBS at room temperature for 2 hours. After washes in PBS/0.3% Triton, sections were labeled with anti-neurofilament heavy chain primary antibody (Abcam) in PBS/0.3% Triton/1% NGS for 48 hours at 4°C with constant agitation. Secondary antibodies conjugated with red fluorochromes (Invitrogen, Alexa Fluor 568) were then incubated for two hours at room temperature. Spinal cord sections were coverslipped with fluorescence mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Analysis of Axons

Axon integrity was measured via the amount of neurofilament fluorescence immunolabeling in the motor tracts of the ventral funiculus in the lumbar spinal cord from 4 animals per group, using 10 sections per animal from the L4 through L6 region. Images adjacent to the anterior median fissure were captured with a fluorescence microscope (Zeiss) and quantified with ImageJ software. Based on DAPI fluorescence, ventral white matter axons were analyzed in normal appearing white matter that was free of extreme DAPI labeling, which indicates an area of immune cell infiltration and would

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be expected to display loss of axons. Analysis of axons included the following steps: (1) medial ventral white matter tracts were sectioned by using the drawing function in ImageJ, (2) the ROI was cropped, (3) the image contrast was adjusted to a preset value (4), and then fluorescence intensity of neurofilament immunolabeling was measured and reported in arbitrary units (AU).

Motor Neuron Analysis

Quantitative analysis of motor neurons was performed by manually counting cresyl violet stained cells in the ventral-lateral sections of the lumbar spinal cord in lamina IX. Morphological identification of α-motor neurons was distinguished by having large multipolar cell bodies and a visible nucleolus. Cell counts were performed using a light microscope (Olympus) and differences between 4 animals per group were compared by averaging 10 sections from each animal. Cell fragments and cells without visible nucleoli were excluded.

Statistical Analysis

Data from daily disease score were analyzed using repeated measures ANOVA. Mann-Whitney U-test was used to detect differences between groups for mean disease score, disease onset, and all histological analyses. Differences between groups are considered statistically significant at p-values of less than .05. Data are presented as means ± SD.

Results

Exercise volume dramatically decreases after EAE onset

The group of C57BL/6J mice with access to running wheels performed $4.85 \pm$ 2.46 kilometers at the peak (day 6 post immunization) and averaged 3.45 ± 1.29 kilometers before the onset of EAE (days 0 through 13). The onset of EAE in the exercise group occurred at 13.75 ± 0.96 days after injections. From day 14 on, the mice only performed 1.12 ± 0.67 kilometers per day until they were euthanized after day 25, which was significantly different from the volume of exercise performed before clinical signs of disease onset (n=4, p<.01) (Fig. 3.1).

Exercise delays the onset of EAE and reduces disease severity

To determine if there were clinical differences between groups, six mice in the EAE sedentary group and four mice in the EAE exercise group were behaviorally tested for disease score on a daily basis using a standard motor disability scoring method. The day when clinical signs and symptoms were first observed was considered the day of EAE onset. MOG induced EAE in sedentary mice displayed earlier clinical signs of disease at 12 ± 0.82 days, whereas EAE mice exposed to voluntary exercise had later disease onset at 13.75 ± 0.96 days post injection (p<.05). Throughout the disease course, EAE mice exposed to exercise continued to have a significantly lower disease score (1.86 ± 0.21) when compared to sedentary counterparts (3.15 ± 0.53; p<.01) (Fig. 3.2). Throughout experimentation, 2 mice in the sedentary group died due to EAE, whereas all mice in the exercise group survived.

Cellular infiltration is attenuated with exercise

Since the primary pathology in MS and EAE is the trafficking of autoreactive cells from the periphery across the BBB, we aimed to determine if voluntary exercise could abrogate the load of infiltrating cells when compared to sedentary animals. In the EAE model, immune cells gain entry through dorsal vessels in the L5 region of the lumbar spinal cord through increased expression of CCL20 through chronic hindlimb sensory activation (Arima et al., 2012). To determine if chronic motor activation could decrease the load of infiltrating autoreactive cells in the ventral region of the lumbar spinal cord, we examined at least 10 representative histology sections from L4 to L6 per animal. Nissl staining revealed large amounts of cellular infiltrates in the sedentary EAE group when expressed as percent area occupied within the ventral white matter tracts (16.6 ± 6.45%; n=4), which was significantly lower in the EAE exercise group (4.46 ± 1.18%, n=4; p<.01) (Fig. 3.3).

Exercise decreases demyelination area in EAE mice

Since demyelination is a hallmark pathological feature of MS and EAE, we were interested in determining if there were differences in demyelination in exercise and sedentary EAE animals. Using luxol fast blue to stain myelinated white matter tracts in the lumbar spinal cord, we analyzed the ventral regions of the L4 through L6 lumbar spinal cord. Analysis revealed that there was a significant loss of myelination in the sedentary group when compared to the exercised animals (n=4/group; p<.01). Luxol fast blue staining occupied 67.34 ± 5.83% of the ventral white matter in the EAE exercise group, and only 47.59 ± 7.73% staining in the EAE sedentary group. Demyelination

appeared to occur in the same regions as immune cell infiltration, with most damage occurring towards the outermost regions of the white matter tracts (Fig. 3.4).

Exercise protects EAE mice from axon loss

Axon loss in MS and EAE is highly related to motor disability. Motor tracts occupy a high density of area in the ventral funiculus of the lumbar spinal cord which includes the medial longitudinal fasciculus, ventral reticulospinal tract, tectospinal tract, and the ventral corticospinal tract. Axons from these tracts innervate interneurons and motor neurons in the ventral grey matter and are responsible for activation and coordination of skeletal muscle motor units. To investigate the axon density in this region of the lumbar spinal cord, we performed immunolabeling with neurofilament heavy-chain antibodies to quantify axon density. Four animals per group were used for analysis with 10 sections analyzed from each animal. Analysis revealed a higher density of axon immunolabeling in the L4 through L6 region of the ventral medial spinal cord in the exercise group when compared to the sedentary group (p<.01) (Fig. 3.6).

Motor neuron loss is reduced with chronic exercise

Motor neurons in the lumbar spinal cord activate skeletal muscle throughout the hindlimb region. (Vogt 2009 Ann Neurol). Loss of motor neurons results in irreversible loss of motor function and exercise has been shown to prevent motor neuron loss and preserve locomotor activity (Grondard et al., 2005). To determine if chronic exercise prior to EAE onset could preserve motor neuron numbers in the lumbar spinal cord, we performed manual counts of α-motor neurons based on morphology in the L4 through L6

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region in four mice per experimental group. We found that the voluntary exercise group had significantly greater numbers of α-motor neurons (10.15 ± 1.05 cells/section), when compared to sedentary EAE animals (5.43 ± 1.11 cells/section, p<.01). Sedentary animals displayed shrunken and fragmented Nissl bodies with no detectable nucleoli in many of the lumbar sections analyzed, while the exercise group showed more intact morphology (Fig. 3.7).

Discussion

Axonal and neuronal injury occurs in both the white and grey matter regions very early in the disease course of people with MS (Dutta and Trapp, 2011; Herz et al., 2010) and in the EAE animal model (Aharoni et al., 2005). Neurological disability in MS and EAE show a causal relationship between lymphocyte infiltration, demyelination, and axon loss (Trapp et al., 1998; De Stefano et al., 2002). Exercise is a behavior that activates molecular and cellular cascades that support CNS function, plasticity, and protection against degeneration (Grondard et al., 2005). Here we provide evidence that exercise can modulate disease onset and severity in the EAE model based on our observations of delayed clinical signs and lower disability scores in exercise compared to sedentary animals.

The differences in disease scores may be a result of decreased immune cell infiltration and subsequent reductions in demyelination, axon and α-motor neuron loss. Our findings are consistent with other observations showing that exercise in EAE animal models has shown to be effective at attenuating disease onset and reducing severity (Rossi et al., 2009; Berchtold et al., 2005; Le Page et al., 1996). For example, Rossi and

colleagues showed that exercise reduced overall clinical disease severity and neurological deficits compared to control animals after EAE induction (Rossi et al., 2009). Exercise can also delay the onset of clinical signs of disease in adoptive transfer of EAE in mice forced to run on treadmills (Le Page et al., 1996).

Our MOG induced C57BL/6J EAE mice showed marked reductions in autoreactive immune cell infiltrates in the lumbar spinal cord. In contrast, Rossi et al. observed no differences in inflammatory infiltration and secondary demyelination and neuronal damage in the spinal cord of exercising and non-exercising EAE mice and suggested that exercise does not affect the inflammatory immune infiltration in EAE, but confers more of a neuroprotective effect (Rossi et al., 2009). Our data show that exercise alters immune cell infiltration into the CNS; however, the mechanisms remain unclear. In a murine asthma model, exercise altered immune cell infiltration to the respiratory vasculature through increased expression of CD4+ T-regulatory cells in the lymph nodes that provided suppressive effects through the production of regulatory cytokines and reductions in IL-17and IL-10 (Lowder et al., 2010). Chronic sensory activation by hindlimb musculature, without chronic exercise training, activates the IL-6 amplifier to upregulate CCL20 in dorsal blood vessel endothelial cells, specifically in the L5 region, which allows entry of CD4+ T-cells. Hindlimb suspension and subsequent deactivation of afferent activation of CCL20 significantly reduces the cellular infiltration (Arima et al., 2012). It would be expected that this mechanism would be highly activated in our exercised animals due to chronic hindlimb activation for approximately 3 hours per day prior to disease onset. This was not the case, as we witnessed large reductions in inflammatory infiltrates in EAE mice exposed to exercise. This may be due to reported

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reductions in IL-17 with chronic exercise, which would interfere with expression of IL-17 dependent upregulation of chemokines (Murakami and Hirano, 2011). This may indicate that chronic motor activation results in mechanisms that would prevent the accumulation and infiltration of autoreactive cells into the lumbar spinal cord. MS treatments that prevent lymphocyte egress from lymphoid organs subsequently inhibit autoimmune inflammatory infiltrates and prevent disability progression in EAE (Choi et al., 2011) and people with MS (Rinaldi et al., 2012). Our data show that exercise may be an effective strategy for reducing pathogenic immune cell responses in MS.

Demyelination in MS is a result of the degeneration of the myelin sheath and loss of oligodendrocytes (Wujek et al., 2002). Autoreactive immune cell infiltrates recognize myelin antigens and subsequently produce reactive oxygen and nitrogen species that create an environment for dissemination of myelin glycoproteins (Li et al., 2011; Haider et al., 2011). We show that demyelination is extreme in sedentary EAE animals, but is significantly reduced in in EAE animals that exercised. This could be due to the differences in immune cell infiltration between the groups. Demyelination is a hallmark sign of pathology in the EAE model, and is shown to gradually become more widespread with increased time after disease onset (Aharoni et al., 2005). Ultrastructural analysis of demyelination utilizing electron microscopy uncovers differences in pathology between several models of EAE. Proteolipid protein-induced EAE in SJL/J mice show dissemination or complete disappearance of the myelin sheath surrounding axons, which is contrasted with the breakdown of the entire axonal arrangement indicative of neurodegeneration with concomitant axon loss in MOG-induced C57BL/6J mice (Aharoni et al., 2005). This is consistent with our finding that areas of demyelination

appear to coincide with axon loss. The only other report to measure demyelination in EAE mice after a period of exercise training showed no differences demyelination between EAE exercise mice and EAE sedentary mice, but found substantial differences in neurophysiological measures and reduced dendritic spine loss (Rossi et al., 2009).

People with MS display loss of axons, which may be a major determinant of progressive neurological disability (Wujek et al., 2002). Axon pathology in EAE has been extensively investigated, and degenerative changes in axons include ion channel redistribution, vesicular transport deficits, Wallerian degeneration, and complete axonal transection (Herrero-Herranz et al., 2008; Herrero-Herranz et al., 2008; Lee et al., 2012; Herz et al., 2010; Linker and Lee, 2009). The molecular mechanisms that lead to axonal loss are not very well known. Some research has shown that mitochondrial dysfunction plays a large role in axonopathology in EAE through the regulation of calcium homeostasis (Forte et al., 2007). This mitochondrial dysfunction is initiated through the production of reactive oxygen and nitrogen species that is produced by infiltrating immune cells and activation of resident glial cells (Li et al., 2011; Gilgun-Sherki et al., 2004; Fischer et al., 2012). Altering mitochondrial homeostasis also leads to cascades that activate apoptotic mechanisms via the release of cytochrome-c (Sajad et al., 2011; Das et al., 2008). Extrinsic apoptotic pathways are also pronounced in axonopathy, with increased TNF-alpha and Fas/FasL induced cytotoxicity. Mice deficient in ciliary neurotrophic factor exhibit more severe clinical disease and greater loss of myelin and axons (Linker et al., 2005). Our data show that axon loss is much more pronounced in sedentary mice when compared to mice exposed to voluntary exercise. This could be mainly due to the more pronounced load of inflammatory infiltrates that lead to

axonopathy, but others have shown that axon degeneration occurs in areas free from immune cell assault such as in normal appearing white matter and the grey matter of the spinal cord and in distant regions within the brain (Herrero-Herranz et al., 2008; Soulika et al., 2009) suggesting that neurodegenerative mechanisms intrinsic to the CNS are at work and persistently contribute to axon pathology, chronic activation of astrocytes and microglia. We also find this to be true, as we focused our axon analysis on regions free from excessive DAPI labeling. Although, we did not determine if there were large amounts of infiltrates and axon transection upstream of the L4 through L6 region, which could contribute to decreased axon immunolabeling in the downstream region analyzed.

Lower motor neuron loss has been implicated as a major determinant of motor disability in MS. Compound muscle action potential amplitudes and motor unit numbers are decreased in people with MS, and lumbar spinal cord autopsy specimens reveal massive loss of α-motor neurons of 48% when compared to non-MS controls (Vogt et al., 2009). In several different animal models of MS, α-motor neuron loss was 47% in C57BL/6 mice and a 74% loss in SJL/J mice (Vogt et al., 2009). It appears as if α-motor neurons undergo apoptosis during the onset of disease or exacerbation with little to no neuronal loss in remission phases. Others have not reported differences in α-motor neuron loss in the EAE model, but show that α-motor neuron cell bodies are 15% more atrophied than control mice and that α-motor neuron dendrites have proximal thinning, shortening, and fragmentation (Bannerman et al., 2005). This latter report may show disparate findings due to that the mean disease score of less than 2.5 initially, which then stabilized below 2. It is possible that the relatively low disease score in that group indicates that α-motor neurons were spared from entering into apoptosis. More recent

reports expose that α-motor neurons are lost as a function of disease course in MOG induced C57BL/6 mice, with a 54% loss of motor neurons after 50 days, which is significantly reduced when treated with glatiramer acetetate, an approved MS drug that is immunomodulatory and neuroprotective (Aharoni et al., 2005). Although absent of large numbers of infiltrates, grey matter regions of the brain and spinal cord have abundant cell loss due to apoptosis (Soulika et al., 2009; Aharoni et al., 2005). By manually counting α-motor neurons in the lamina IX regions of the L4 through L6 lumbar spinal cord, we determined that motor neuron loss in sedentary mice was much more pronounced than in mice that were exposed to exercise.

In conclusion, our data suggest that chronic aerobic exercise during the development of EAE, prior to disease onset, with minimal exercise during EAE, attenuates the clinical severity of the autoimmune disease. Exercise appears to modulate components of the immune response that lead to decreased transit of autoreactive cells across the BBB into the lumbar spinal cord. It is not known if the reductions in cellular infiltration alone lead to the decreased neuropathology or if there are also intrinsic mechanisms that become activated and resist degeneration. Exploring the neuropathology of exercise in the EAE model is important for determining the efficacy of exercise as a possible treatment for MS. Future studies should delineate the mechanisms by which exercise may be neuroprotective in MS.

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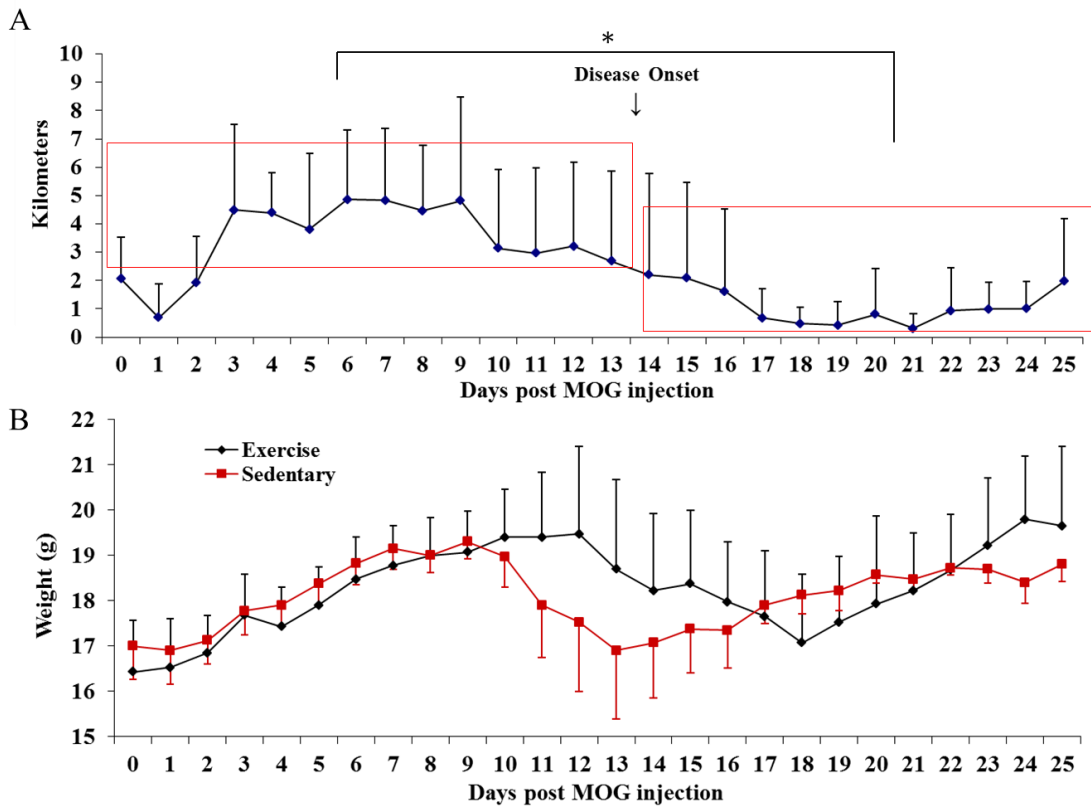
Figure 3.1. Daily distance performed and weight loss associated with EAE onset. Mice were injected with MOG on day 0. (A) Distance was recorded at approximately the same time each morning. Exercise volume was significantly reduced after disease onset (day 13.75 ± 0.96) (*p<.01, n=4). (B) Weight loss coincides with EAE onset. No significant weight differences were found between EAE animals exposed to exercise or sedentary conditions. Data are means $\pm$ SD; n=4/group.
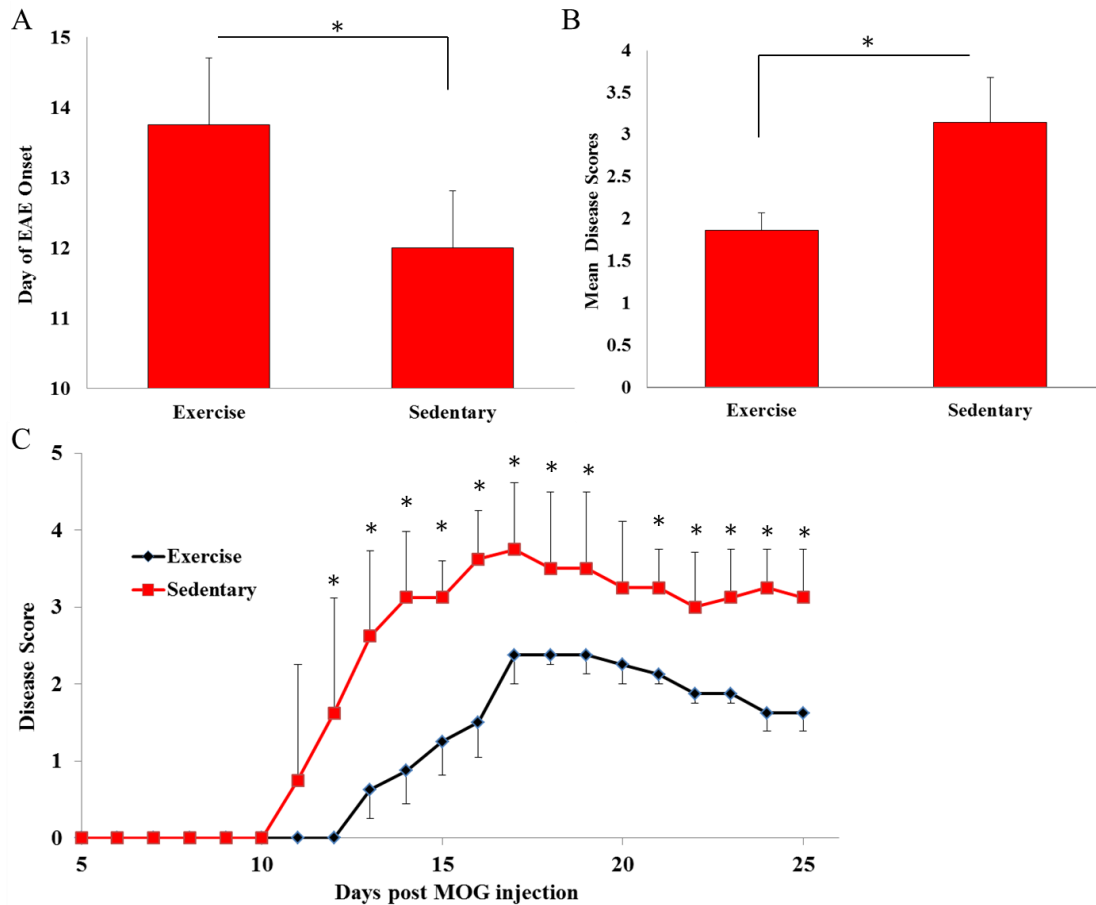
Figure 3.2. Clinical disease course of MOG induced EAE mice. Mice were monitored daily and scored as described in Methods. (A) Day of disease onset was assessed via the first clinical motor disabilities recorded for each animal. Onset was 1.75 days earlier in the sedentary group (n=4; *p<.05). (B) Differences between mean disease score in exercise and sedentary groups were averaged starting at disease onset. Data are means ± SD; n=4/group; *p<.01. (C) Daily clinical scores are shown for mice exposed to exercise or sedentary conditions. Data are means ± SD; *p<.05; n=4/group.

Exercise EAE   Sedentary EAE

A

B

C
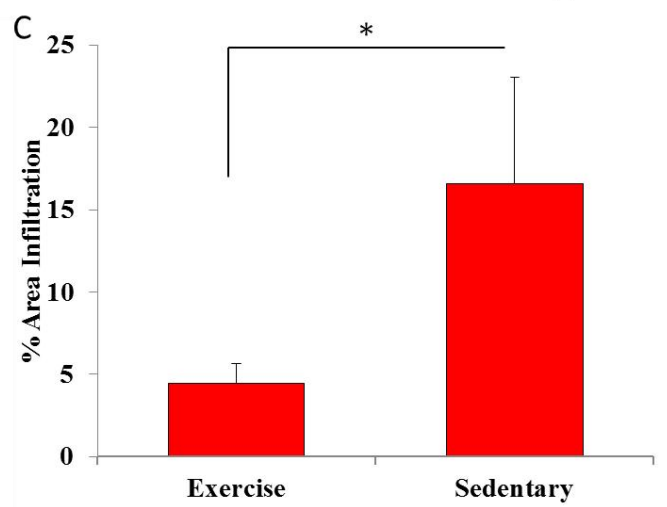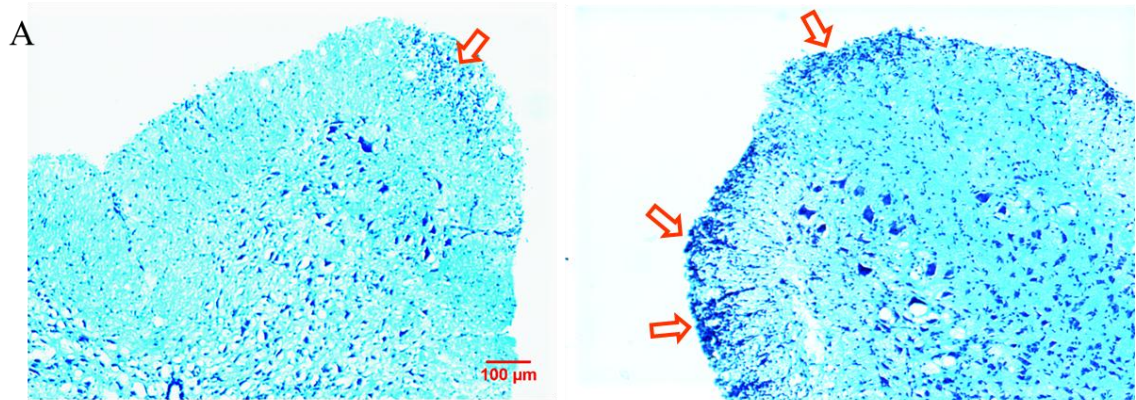
Figure 3.3. Cellular infiltration is more severe in sedentary mice with MOG induced EAE. (A) Cresyl violet staining of cellular infiltrates in the ventral region of the lumbar spinal cord in exercise and sedentary animals with EAE. (B) Ventral white matter tracts were segregated from the images above and converted to binary images for quantification. (C) Quantification comparing the means of at least 10 sections from each animal. Data are means ± SD; *p<.01; n=4/group.
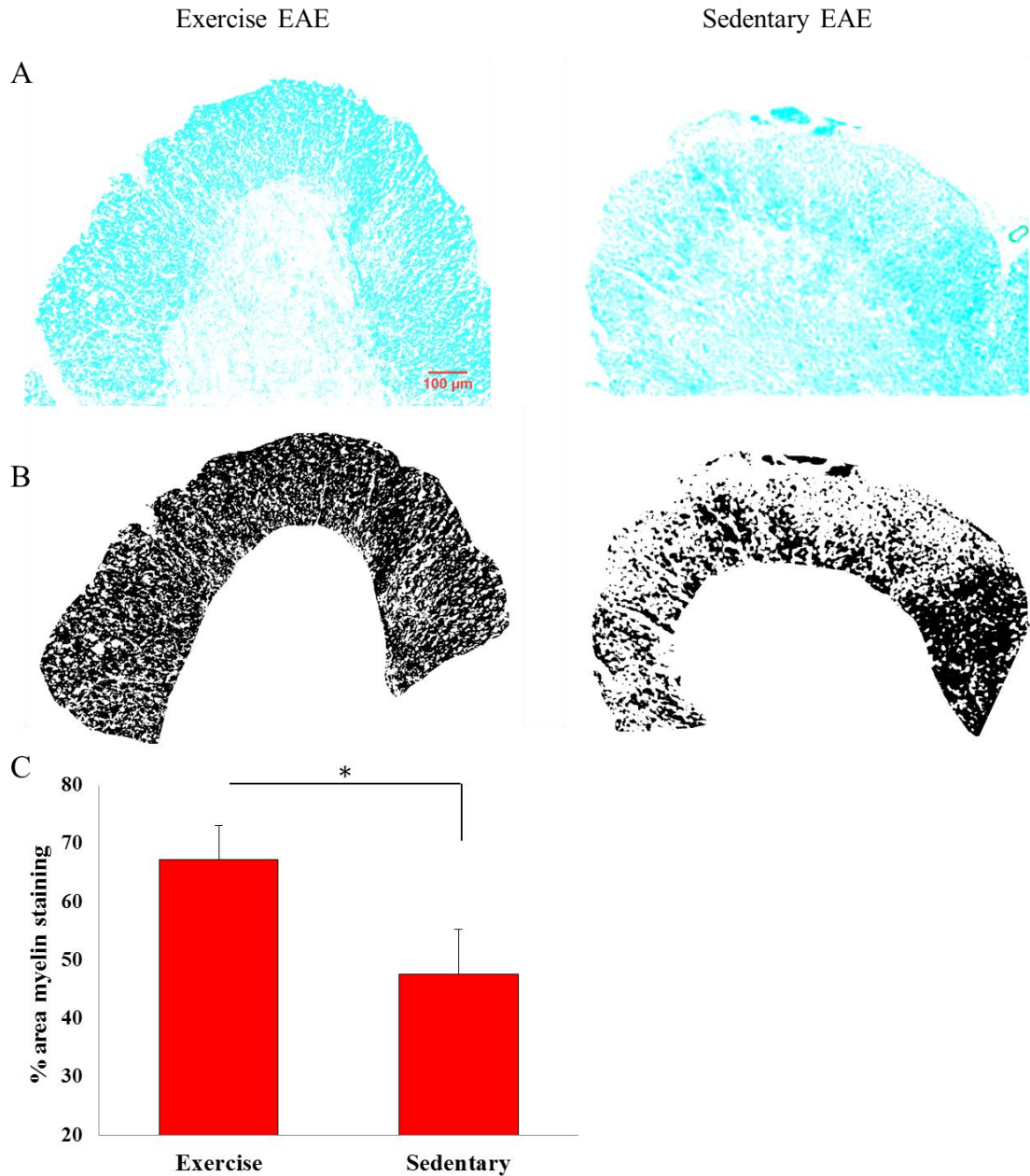
Figure 3.4. Exercise protects against demyelination. (A) Luxol fast blue staining of the ventral white matter of an L4 lumbar spinal cord section of animals exposed to exercise or sedentary conditions. (B) Binary images of myelin staining from above images. (C) L4 through L6 myelin staining was quantified and expressed as a percent of total area (black area/total area). Data is expressed as means ± SD; *p<.01; n=4/group.
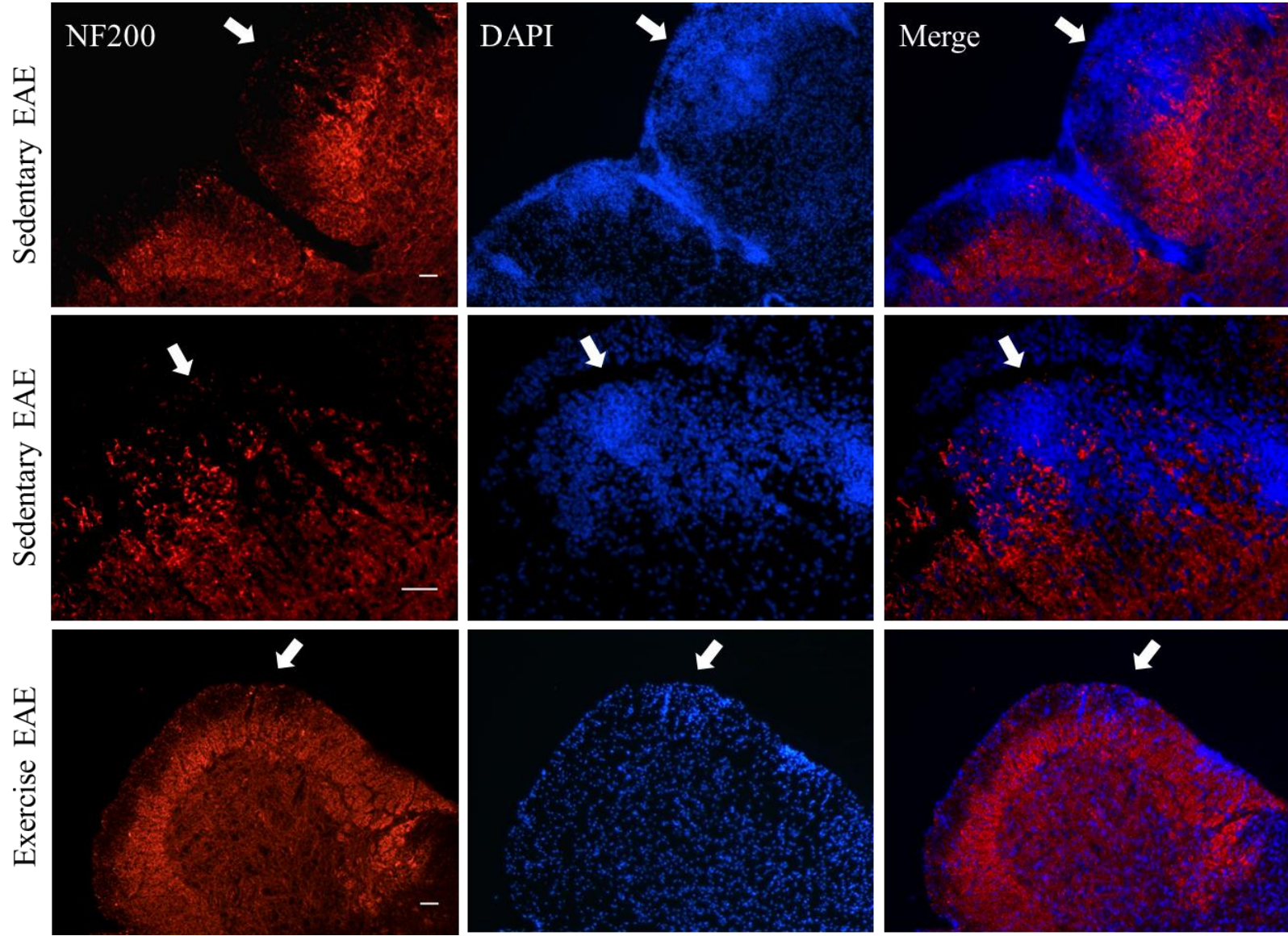
Figure 3.5. Cellular infiltration creates voids in neurofilament immunolabeling. Neurofilament heavy-chain and DAPI representative images from EAE animals exposed to exercise or sedentary conditions. Arrows indicate areas of autoimmune lesions with loss of neurofilament immunolabeling and extreme DAPI fluorescence indicative of cellular infiltration. Merged images show the coincidence with infiltration and loss of axons. The EAE animals exposed to exercise show smaller areas of infiltrates and minor loss of neurofilament immunolabling when compared to sedentary animals with EAE. Scale bar represents 50um.

Exercise EAE   Sedentary EAE

30 µm

A

B

C
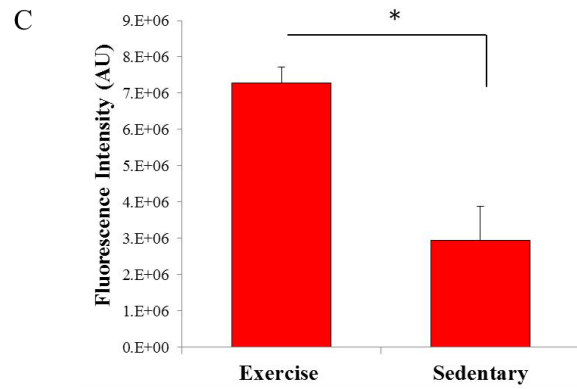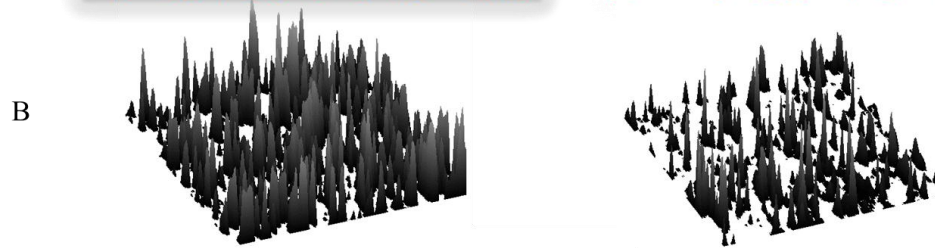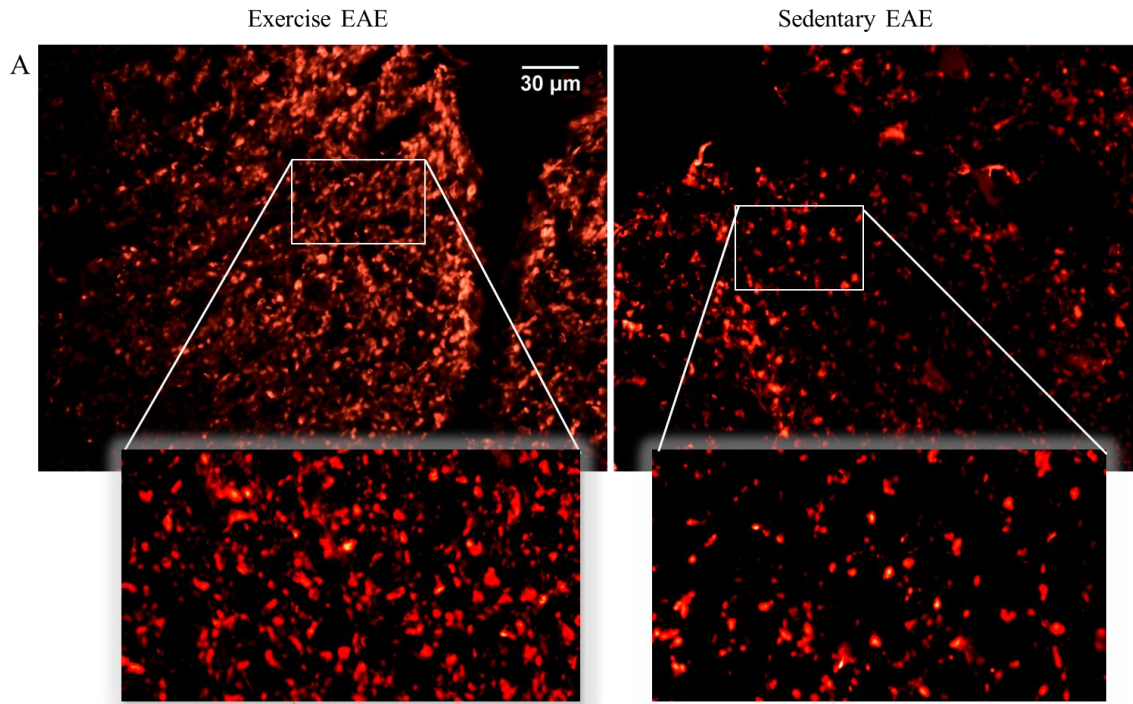
Figure 3.6. Neurofilament immunolabeling reveals axon loss in the motor tracts of the anterior funiculus of the lumber spinal cord. (A) Images from the medial anterior funiculus of the lumbar spinal cord in an EAE animal exposed to chronic exercise and a sedentary animal with EAE. High magnification images of the medial ventral efferent tracts showing a higher density of axon immunolabeling in the EAE animals exposed to exercise compared to sedentary conditions. (B) Surface plots of fluorescence intensity from representative images above. (C) Quantification of axon immunolabeling fluorescence intensity from the L4 through L6 medial ventral region. Data are means ± SD; *p<.01; n=4/group.
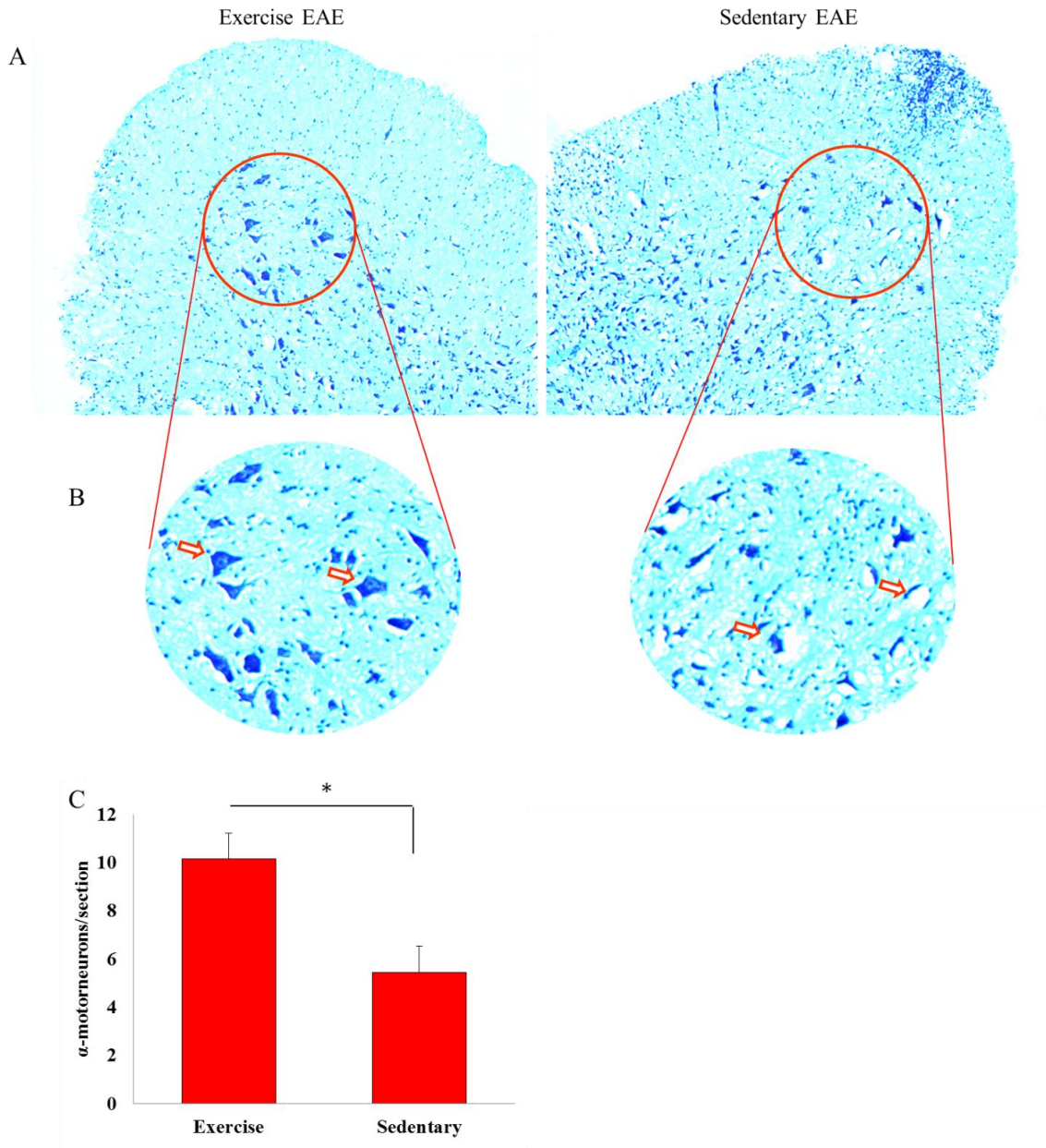
Exercise EAE   Sedentary EAE

Figure 3.7. Exercise protects EAE mice from motor neuron loss. (A) Representative images of motor neuron loss in lamina IX of L4 through L6 sections from EAE animals exposed to exercise or sedentary conditions. (B) High magnification images of motor neurons showing preserved α-motor neuron morphology in EAE animals exposed to exercise and shrunken Nissl bodies and loss of nucleoli in sedentary EAE animals. (C) Lamina IX α-motor neurons were manually counted in sections from L4 through L6 from each animal. 46.5% fewer viable α-motor neurons were counted in EAE animals exposed to sedentary condition. Data are means ± SD; *p<.01; n=4/group.

CHAPTER 4

EXERCISE ACTIVATES PRO-SURVIVAL PATHWAYS IN EXPERIMENTAL

AUTOIMMUNE ENCEPHALOMYELITIS[2]

Pryor, B., Edwards, G., Binkowski, J., White, L. To be submitted to *Neuroscience*

Abstract

Multiple Sclerosis (MS) is an autoimmune disease affecting the central nervous system that results in continued loss of cognitive, sensory, and motor function. In this study we aimed to evaluate the effects of voluntary exercise on the activation of pro-survival pathways in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. C57BL/6J mice were injected with an emulsion containing myelin oligodendrocyte glycoprotein (MOG) and then randomized to housing with a running wheel or a locked wheel. EAE mice exposed to exercise displayed less severe neurological disease score and later onset of disease when compared to sedentary EAE animals. No differences were observed between TrkB receptors in the L4 through L6 region of the spinal cord, but phosphorylated (Y515) TrkB receptors were significantly increased in the exercise group when compared to sedentary EAE animals. Expression of mitochondrial outer membrane pro-survival members Bcl-2, Bcl-XL, and Mcl-1 were all significantly higher in the EAE animals exposed to exercise. These data suggest that chronic voluntary exercise activates intrinsic pathways that lead neuroprotection.

Keywords: Multiple sclerosis, experimental autoimmune encephalomyelitis, exercise, neuroprotection, pro-survival

Introduction

Multiple sclerosis (MS) is a T-cell mediated autoimmune disease that targets the myelin glycoproteins surrounding axons in the central nervous system (CNS) (McFarland and Martin, 2007). MS is the leading cause of neurologic disability in young adults, affecting approximately 400,000 individuals in the USA and 2.5 million people worldwide (Ramagopalan et al., 2010; Richards et al., 2002). MS is most often diagnosed in those between the ages of 20 and 40 with a male-to-female ratio of 1:2 (Ramagopalan et al., 2010). Autoimmune infiltration into the CNS results in the development of lesions that accumulate over time, and leads to loss of axonal processes and progressive loss of grey matter. It is this loss of the grey matter that leads to the progressive cognitive and physical disability that individuals with MS experience (Fisniku et al., 2008; Peterson et al., 2001).

The loss of grey matter seen in MS and the animal model of MS, experimental autoimmune encephalomyelitis (EAE), is primarily due to apoptosis (Qin et al., 2010; Aharoni et al., 2011). Apoptosis, a.k.a. programmed cell death, is an evolutionary conserved and genetically regulated process that is critical for normal development. Any defects in the cell death machinery may contribute to pathologies ranging from oncogenesis to autoimmunity to neurodegeneration (Putcha et al., 2003) and is accelerated in neuropathologies such as MS (Dowling et al., 1997). Apoptosis can be divided into two categories: the intrinsic and extrinsic pathways. The intrinsic pathway results in the release of cytochrome-c and other mitochondrial proteins such as DIABLO, with both pathways resulting in the cleavage and activation of caspases. Regulation of apoptosis is determined by the Bcl-2 family of proteins. The Bcl-2 family consists of a

number of evolutionarily conserved sequences that modulate apoptosis through mitochondrial membrane permeability (Cory et al., 2003). Humans and mice have 13 structural homologues of Bcl-2 family proteins (Youle and Strasser, 2008). The Bcl-2 family consists of three groups based upon homology and function. Pro-survival members include Bcl-2, Bcl-XL, and Mcl-1, and pro-apoptotic proteins include Bax, Bak, Bad, Bik, Bid, Bim, and Puma. Interactions between pro-apoptotic and anti-apoptotic Bcl-2 family members controls cells fate by forming heterodimers, with the ratio of pro to anti-apoptotic proteins determining death or survival (Datta et al., 1997). The pro-survival members exert anti-apoptotic activity by binding to and antagonizing the death-promoting members. This results in inhibition of mitochondrial outer membrane pore formation and subsequent activation of executioner caspases (Cory et al., 2003). These anti-apoptotic proteins can be stimulated by cytokines, growth factors, and neurotrophins.

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family that provides trophic support to neurons leading to increased neuronal growth, function and survival. Mature BDNF binds to tropomyosin-related kinase-B (TrkB) transmembrane spanning receptors which are commonly expressed on neurons and glia (Volosin et al., 2006). *In vitro* experiments reveal that when neurons are deprived of trophic support they undergo apoptotic cell death (Kirkland et al., 2010). BDNF can promote cell survival through activation of the TrkB receptor, which transduces several cell signaling pathways including the phosphatidylinositide-3'-OH kinase (PI3K)/Akt pathway. Akt phosphorylates the BCL-2 family member, BAD, which allows BAD to be sequestered by the 14-3-3 protein and inhibits the cascade of events that lead to mitochondrial membrane permeability and ultimately, apoptosis (Datta et al., 1997). The

BDNF/PI3K/Akt pathway also regulates apoptosis by suppressing the expression of death genes by phosphorylating FOXO1 transcription factors (Bruel-Jungerman et al., 2009; Biggs et al., 1999). BDNF rescues cells from death through increases in pro-survival Bcl-2 mRNA levels in a time-dependent manner and inhibits the mitochondrial depolarization induced by serum starvation induced cell death (Kajiya et al., 2009).

Activity-dependent Ca^{++} influx through L-type voltage sensitive Ca^{++} channels stimulates transcription from BDNF promoter III via a CREB family dependent mechanism (Tao et al., 1998). BDNF expression is regulated at several levels and functions as a modulator of neuronal plasticity and a mediator of neuronal survival and differentiation. Neeper and colleagues were the first group to show that BDNF is upregulated in the hippocampus and neocortex in rats with free access to running wheels. Levels of BDNF increased with length of exposure to exercise and showed a positive correlation with distance run per night (Neeper et al., 1995). Gene microarray analysis of rodent hippocampus exposed to voluntary exercise displays that BDNF is the only trophic factor whose gene was consistently expressed at all exercise timepoints measured (Molteni et al., 2002). It is now well recognized that exercise increases BDNF/TrkB signaling in the CNS and leads to expression of genes involved in plasticity and apoptosis in spinal motor neurons, dopaminergic neurons, and leads to the activation of cell survival pathways (Keeler et al., 2012; Wu et al., 2011; Chen and Russo-Neustadt, 2007). BDNF activation of TrkB receptors greatly increases Bcl-2 mRNA levels and mitochondrial membrane potential (Kajiya et al., 2009) and may be the mechanism by which exercise is neuroprotective in ischemia and traumatic brain injury (TBI) (Liebelt et al., 2010; Kim et al., 2010).

The aims of this study were to determine whether voluntary exercise in C57BL/6J mice alters TrkB expression and phosphorylation in myelin oligodendrocyte glycoprotein (MOG) induced EAE. As exercise leads to activation of TrkB signaling cascades, which has been shown to increase pro-survival Bcl-2 family members *in vitro*, we aimed to determine if exercise can modulate expression of pro-survival members and prevent apoptosis *in vivo* in MOG induced EAE. Here we describe that voluntary exercise in C57BL/6J mice attenuates clinical disease onset and severity in MOG induced EAE. We also present evidence that TrkB receptors are highly phosphorylated and the pro-survival members Bcl-2, Bcl-XL, and Mcl-1 have increased expression in animals exposed to exercise prior to and during EAE onset when compared to those exposed to sedentary conditions.

Experimental Procedures

Experimental Animals

C57BL/6J mice (Jackson Laboratories), 16-20g, 10-weeks of age, were housed singly in a 12:12 hour light/dark cycle in a temperature-controlled environment (22 °C; 50–60% humidity). Standard laboratory irradiated chow (Purina PicoLab Rodent Diet 20) and water were available ad libitum. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

EAE Induction

Twenty animals were induced with EAE by injecting 200 µg of myelin oligodendrocyte glycoprotein 35-55 (MOG 35-55) (Biomatik) in 200 microliters of

complete Freunds adjuvant (CFA) containing 1 mg/mL heat-killed *Mycobacterium*

tuberculosis (MP Biomedical). The CFA was supplemented with mortar-ground, heat-

killed *Mycobacterium tuberculosis* (Difco H37Ra) for a total concentration of 5mg/mL.

The MOG/CFA emulsion was injected subcutaneously at two sites; one on the flanks and

one behind the neck (100µL each). 400 nanograms (800ng total) of *Bordatella pertussis*

toxin was injected intraperitoneally on the MOG/CFA injection day (day 0) and again 24

hours later (day 1). Animals that were injected but showed no behavioral disability and

no histological signs of autoimmune infiltrates were used as non-EAE sedentary controls.

Disability Scoring and Monitoring

All mice were scored daily for EAE using a standard 6-point disability scale

(Beeton et al., 2007). (0 = no disease; 1 = limp tail; 2 = mild paraparesis, ataxia; 3 =

moderate paraparesis; 4 = complete hind limb paralysis; 5 = complete hind limb paralysis

and incontinence; 6 = moribund, difficulty breathing, does not eat or drink).

Exercise Protocol

Animals were randomly assigned to either an EAE voluntary exercise group (n=8)

or EAE sedentary group (n=12). Animals in the exercise group were individually housed

and had access to a running wheel within their cage 24 hours/day, 7 days/week. The

sedentary group was exposed to the same environment, but the running wheels were

locked. An electronic system was used to measure running distance, time, and average

velocity performed in a 24-hour period.

Immunohistochemistry

After 25 days, mice were euthanized with CO_2 and perfused transcardially with PBS followed by 4% paraformaldehyde. Spinal cord sections were postfixed in 4% paraformaldehyde for four hours then 20% sucrose solution for 24 hours. Lumbar spinal cords from four mice per group were cut at 20 μm coronal sections on a cryostat at -20 °C. Sections were blocked with 10% normal goat serum (NGS) in 0.3% Triton-X in PBS at room temperature for 2 hours. After three washes in PBS/0.3% Triton, sections were labeled with primary antibodies in PBS/0.3% Triton/1% NGS for 48 hours at 4°C with constant agitation. Primary antibodies were chicken anti-neurofilament heavy chain (Abcam), rabbit anti-TrkB receptor (Santa Cruz Biotechnology), and rabbit anti-TrkB receptor phospho-Y515 (Abcam). Fluorochrome conjugated secondary antibodies were then incubated for two hours at room temperature (Alexa Fluor 488 and 568, Invitrogen). Spinal cord sections were direct mounted on charged slides and coverslipped with fluorescence mounting medium containing DAPI (Vector Laboratories).

Western Blot Analysis

After 25 days, mice were euthanized with CO_2 and spinal cords were removed and rapidly frozen in liquid nitrogen. Frozen spinal cord sections from four mice per group were homogenized in 30μl/mg of RIPA lysis buffer containing 10μl/mL protease and phosphatase inhibitor cocktail (Halt, Thermo Scientific). Protein concentrations were determined by modified Lowry method protein assay (BioRad). Spinal cord homogenates were subjected to SDS-PAGE on 8-15% gels and transferred to Polyvinylidene fluoride membranes. After blocking for 1 hour at room temperature with 5% bovine serum

albumin, the membranes were incubated overnight at 4°C with rabbit antibodies against

TrkB receptor (gp145trkB) (Santa Cruz Biotechnology), TrkB receptor phospho-Y515

(Abcam), Bcl-2, Bcl-2 phospho-S70, Bcl-XL, and Mcl-1 (Cell Signaling Technology).

Secondary antibodies were HRP conjugated (Cell Signaling Technology) and

immunodetection was assessed by using an enhanced chemiluminescence detection kit

(GE Healthcare/Amersham Biosciences). Densitometry was assessed with ImageJ

software (ImageJ 1.44p).

Statistical Analysis

      Data from daily disease score were analyzed using repeated measures ANOVA.

Differences between groups for mean disease score, disease onset, and western blot

densitometry were analyzed with Mann-Whitney *U*-test. Differences between groups are

considered statistically significant at p-values of less than 0.05. Data are presented as

means ± SD.

Results

Exercise volume is attenuated after EAE onset

      The group of C57BL/6J mice with access to running wheels performed 5.5 ± 4.3

kilometers at the peak (day 12 post immunization) and averaged 4.4 ± 1.3 kilometers

before the onset of EAE (days 0 through 14). The onset of EAE in the exercise group

occurred at 14.3 ± 0.6 days after injections. From day 14 on, the mice only performed 0.5

± 0.7 kilometers per day until they were euthanized after day 25, which was significantly

different from the volume of exercise performed before clinical signs of disease onset

(p<.01; n=8) (Fig. 4.1).

Exercise delays the onset of EAE and reduces disease severity

To determine if there were clinical differences between groups, eight mice per

group were behaviorally tested for disease severity on a daily basis for 25 days post

MOG/adjuvant injection using a standard motor disability scoring method (Beeton et al.,

2007). The day when clinical signs and symptoms were first observed was considered the

day of onset. MOG induced EAE in sedentary mice displayed earlier clinical signs of

disease at 12.3 ± 0.5 days, whereas EAE mice exposed to voluntary exercise had later

disease onset at 14.3 ± 0.6 days post injection (p<.01). Throughout the disease course,

EAE mice exposed to exercise continued to have a significantly lower disease score ($2.63

\pm 0.35$) when compared to sedentary counterparts (3.67 ± 0.37; p<.01) (Fig. 4.2).

Exercise leads to TrkB receptor activation in EAE mice

We hypothesized that exercise may have an effect on total TrkB concentration in

L4 through L6 lumbar spinal cords. We performed western blot analysis on spinal cord

homogenates and found no significant differences between EAE exercise and EAE

sedentary mice (p=.89), EAE exercise and non-EAE sedentary (p=.08), or EAE sedentary

and non-EAE sedentary (p=.25; n=4/group) (Fig. 4.3). Since we have previously

determined that α-motor neurons are lost in EAE sedentary mice when compared to EAE

exercise animals, we performed immunohistochemistry in the L4-L6 region to determine

if TrkB receptors are present on motor neurons in these groups. We found that TrkB

immunolabeling was ubiquitously present on motor neurons and surrounding parenchyma

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in the grey matter region in all groups examined. Co-localization with immunolabeling against neurofilament heavy chain, which is primarily expressed in axons, shows that TrkB receptors are also highly prevalent on axons in the lumbar spinal cord grey matter (Fig. 4.4).

Activation of trophic cascades are initiated when BDNF binds TrkB monomers, leading to dimerization and autophosphorylation of the cytoplasmic domains. We next examined if TrkB phosphorylation of Y515 was increased in EAE mice exposed to exercise relative to sedentary conditions. In the EAE exercise group, we found that TrkB phosphorylation increased several fold over EAE sedentary and non-EAE sedentary mice ($p<.05$; n=4/group) (Fig. 4.5). This reveals that BDNF and/or neurotrophin-3 was highly expressed in the exercise group, even after a period of EAE onset and lower exercise volume.

Infiltrating immune cells have been shown to release BDNF and activate TrkB receptors in areas if infiltrating lesions and this may be explanation for our western blot results (De Santi et al. 2009). To determine if phosphorylation of TrkB receptors occurred in α-motor neurons, we performed immunohistochemistry to show that TrkB receptors are phosphorylated in motor neurons, their axonal processes, and throughout all of the grey matter of the lumbar spinal cord in EAE mice exposed to exercise. In contrast, sedentary mice with EAE showed very low phospho-TrkB immunoreactivity, although the distribution throughout the grey matter appeared to be similar (Fig. 4.6).

EAE mice exposed to exercise have increased pro-survival member expression

Recent evidence suggests that exercise prevents apoptosis in animal models of Alzheimer's and spinal cord injury through increased pro-survival pathway activation with subsequent decreases in pro-apoptotic pathways (Keeler et al., 2012; Um et al., 2011). No studies have determined if the three known mitochondrial pro-survival regulators are altered by exercise in the EAE animal model. We performed western blot analysis on lumbar spinal cord homogenates and found that Mcl-1, Bcl-XL, and BCL-2 expression was altered by exercise in EAE animals. Mcl-1, which is a PI3K dependent regulator of apoptosis, was increased several fold in EAE exercise animals when compared to EAE sedentary animals and sedentary controls (p<.05; n=4/group). Expression of Mcl-1 was almost completely lost in the sedentary EAE group (Fig. 4.7).

Bcl-XL is also transcriptionally upregulated by growth factors and interacts with the pro-apoptotic protein Bax, which prevents large oligomer formation that permeablizes the outer mitochondrial membrane. Analysis of lumbar spinal cord homogenates revealed that Bcl-XL expression was significantly higher in the EAE exercise group compared to EAE sedentary (p<.05), and non-EAE sedentary controls (p<.05). Bcl-XL was also significantly lower in the EAE sedentary group when compared to the non-EAE sedentary controls (p<.05; n=4/group), showing that there is a loss of Bcl-XL expression during EAE in sedentary animals that is elevated above non-EAE sedentary controls when EAE animals are exposed to exercise (Fig. 4.8).

Bcl-2 is pro-survival in response to an array of apoptotic stimuli and overexpression of Bcl-2 confers greater resistance to apoptotic cell death (Murphy et al., 2000; Hata et al., 1999). Bcl-2 forms and heterodimers with pro-apoptotic members

BAX, BAD and BAK and thus inhibits mitochondrial outer membrane permeabilization (Youle and Strasser, 2008). We found that total Bcl-2 protein is highly expressed in the L4-L6 lumbar spinal cord of EAE exercise animals, and is significantly reduced in sedentary EAE animals (p<.05). Similar to Bcl-XL, Bcl-2 is significantly increased when compared to non-EAE sedentary animals (p<.05), while there is a significant loss of Bcl-2 expression in the EAE sedentary animals when compared to the non-EAE controls (p<.05; n=4/group) (Fig. 4.9). Phosphorylation of Bcl-2 on serine 70 (S70) causes dissociation from pro-apoptotic members and disables the pro-survival activity of Bcl-2 (Wei et al., 2008). We found no significant differences in Bcl-2 S70 between any of the groups analyzed (Fig. 4.10).

Discussion

Exercise is a behavior that activates molecular and cellular cascades that support CNS function, plasticity, and protection against degeneration (Grondard et al., 2005). Exercise can lead to neuroprotection from traumatic brain injury (TBI) (Kim et al., 2010; Itoh et al., 2011), ischemia (Sim et al., 2005), neurotoxin insult (Carro et al., 2001), Alzheimer's disease (Um et al., 2011), and Parkinson's disease (Wu et al., 2011). How exercise works to promote neuroprotection in animal models of neurological disease remains unclear, and more emphasis on the topic of exercise as a serious therapeutic treatment is needed. Here we provide evidence that voluntary exercise can delay disease onset and severity in the EAE model. The differences in disease scores may be a result of decreased immune cell infiltration, and subsequent alterations in demyelination, axon and α-motor neuron loss as seen in previous data from our lab (unpublished data). Our

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findings are consistent with observations showing that exercise in EAE animal models is effective at attenuating disease onset and reducing severity (Berchtold et al., 2005; Rossi et al., 2009; Le Page et al., 1996). Rossi et al. discussed that exercise does not alter the inflammatory component of EAE, but exerts a direct neuroprotective effects through intrinsic mechanisms (Rossi et al., 2009). Our findings suggest that the intrinsic mechanisms that lead to neuroprotection with chronic voluntary exercise may be due to activation of trophic pathways and increased expression of pro-survival Bcl-2 family members that inhibit mitochondrial pore formation.

Phosphorylation of the tyrosine domains of TrkB receptors creates docking sites for adapter protein which activate several signaling cascades including the PI3K/Akt, PLCγ/PKC, and the Ras-MAPK/Erk signaling cascades that lead to the activation of transcription factors that are involved in cell survival, growth, and a positive feedback increase in transcription of BDNF (Tao et al., 1998). The level of phosphorylation of the kinase domains of the TrkB receptor is highly correlated with the availability of BDNF, which is upregulated in response to exercise (Berchtold et al., 2005). Exercise has been shown to promote increases in TrkB expression and phosphorylation (Skup et al., 2002; Liu et al., 2008). Phosphorylated TrkB receptors are increased in the hippocampus after four weeks of exercise (Liu et al., 2008) and ablation of TrkB receptors in neural progenitor cells can inhibit exercise-induced increases in hippocampal cell proliferation and neurogenesis (Li et al., 2008). Macias and colleagues found that BDNF mRNA was increased in the lumbar spinal cord ventral horn neurons and oligodendrocytes within the spinal grey matter after 28 days of treadmill training, but with no changes in TrkB expression (Macias et al., 2007). Using double labeling methods and co-localization, we

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find that TrkB receptors are expressed on α-motor neuron perikarya and the axonal processes. Western blots show that there are no significant differences in TrkB expression in the lumbar spinal cord, which is similar to findings by Macias et al. (Macias et al., 2007). We then showed that phosphorylation of TrkB receptors at Y515 was increased in exercised EAE animals several fold over sedentary EAE mice and sedentary control animals. Activation of TrkB receptors on motor neurons activate pathways leading to inhibition of apoptosis in the L4 through L6 region. As we have previously found that α-motor neurons are lost in sedentary mice with EAE when compared to EAE mice exposed to exercise, we then examined if phosphorylation of TrkB receptors are occurring in α-motor neurons. We performed immunohistochemistry and observed that indeed there are high levels of phospho-TrkB expression on the motor neurons and throughout all of the grey matter of the lumbar spinal cord in EAE mice exposed to exercise, suggesting that this neuroprotective pathway is activated. In contrast, sedentary mice with EAE showed very low immunoreactivity for phosphorylated TrkB throughout the grey matter, revealing that the apparent difference between groups is occurring in the region of interest and this loss of trophic activation may lead to increased apoptosis in sedentary EAE mice.

Activation of TrkB receptors by BDNF leads to phosphorylation of PI3K which modulates other downstream pathways that regulate apoptosis through sequestration of pro-apoptotic members to the cytosol and recruitment of pro-survival members to the mitochondria where they interact to prevent pore formation (Putcha et al., 1999; Ishrat et al., 2012). Other ways in which BDNF/TrkB signaling can regulate apoptosis is through transcriptionally regulated mechanisms. PI3K regulated transcription of pro-survival

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members occurs simultaneously with downregulation of pro-apoptotic members, leading to the prevention of cytochrome-c release, caspase activation, and subsequent apoptosis in neurons (Wang et al., 2012).

Bcl-2 is pro-survival in response to an array of apoptotic stimuli through inhibition of mitochondrial cytochrome-c release (Murphy et al., 2000). Mice overexpressing Bcl-2 show greater resistance to apoptotic cell death after ischemia and mice deficient in Bcl-2 have severe apoptotic cell death after ischemic challenge (Hata et al., 1999). In animal models of focal ischemia and TBI, expression of the pro-apoptotic member Bax increases and pro-survival Bcl-2 decreases, leading to increased cell death of neurons (Kim et al., 2010; Sahin et al., 2010). Exercise pre-conditioning increases the expression of Bcl-2 and decreases expression of Bax after ischemia and TBI resulting in the inhibition of neural and glial apoptotic cell death (Liebelt et al., 2010; Kim et al., 2010). We observed that total Bcl-2 protein is increased in the L4-L6 lumbar spinal cord when compared to sedentary EAE animal as well as sedentary non-EAE animals. This is an interesting finding in that exercise preconditioning prior to EAE onset with minimal activity during disease onset leads to greater Bcl-2 expression, while sedentary EAE animals display a loss of Bcl-2 protein content when compared to non-EAE animals. Evidence suggests that phosphorylation Bcl-2 at serine 70 (S70) disables its pro-survival activity in mitotic cells (Wei et al., 2008; Terrano et al., 2010). No differences were found in phosphorylated Bcl-2 levels suggesting that modifications to Bcl-2 do not contribute to group differences, but total Bcl-2 expression may be more important at this stage of disease.

Similar to other Bcl-2 family members, Mcl-1 localizes to the mitochondrial membrane and interacts with pro-apoptotic Bcl-2 family members by forming heterodimers, which inhibits apoptosis induced by several types of cytotoxic stimuli (Yang et al., 1995; Sato et al., 1994; Zhou et al., 1997). Mcl-1 inhibits apoptosis through interactions with several pro-apoptotic Bcl-2 family members including PUMA, Bim-EL, Noxa and BMF (Youle and Strasser, 2008; Chen et al., 2005). Mcl-1 is required for neural development and loss of Mcl-1 in neuronal progenitor cells results in widespread apoptosis, showing that Mcl-1 is required for the survival of newborn neurons. In addition, the loss of Mcl-1 sensitizes neurons to apoptosis induced by DNA damage, while maintenance of high Mcl-1 levels protects neurons against death (Arbour et al., 2008). Our data show that there is almost complete loss of Mcl-1 expression in sedentary EAE animals, while the EAE exercise group expresses Mcl-1 at significantly greater levels than sedentary mice with EAE and sedentary non-EAE animals. Mcl-1 appeared to be extremely responsive to the exercise paradigm, which may not come as a surprise as Mcl-1 is rapidly transcribed via the PI3K/Akt dependent pathway (Wang et al., 1999). Upon apoptosis induction, Mcl-1 is rapidly degraded (Nijhawan et al., 2003). We show that Mcl-1 is almost completely lost in the sedentary EAE group, which may represent an overwhelming apoptotic stimulus.

The antiapoptotic mammalian Bcl-2 family protein Bcl-XL, localizes to the mitochondrial membrane and is required for normal development of the CNS (Motoyama et al., 1995). Bcl-XL regulates cell survival by at least two distinct mechanisms; one is associated with heterodimerization with the pro-apoptotic protein Bax, which prevents large oligomer formation that permeabilizes the outer mitochondrial membrane, and the

other with the ability to form a sustained ion channel that maintains normal membrane polarization (Minn et al., 1999). By out-competing Bax for the interactions leading to membrane permeabilization, Bcl-XL binds Bax, thus inhibiting the binding of Bax to the outer mitochondrial membrane and subsequently forming oligomeric pores (Billen et al., 2008). Similar to Bcl-2, Bcl-XL is transcriptionally induced by growth factors (Grad et al., 2000). In a streptozotocin-induced diabetes animal model, exercise training significantly increased Bcl-XL expression and decreased apoptosis in cardiac myocytes when compared to sedentary diabetic animals (Cheng et al., 2012). Our results show that exercise in EAE animals significantly increases Bcl-XL when compared to sedentary EAE or non-EAE animals. This again displays that sedentary animals with EAE lose pro-survival protein expression, while EAE animals exposed to exercise increase Bcl-XL levels to greater than that of the non-EAE animals.

In conclusion, we show that exercise beginning during adjuvant administration, before the onset of disease, leads to attenuated disease onset and reduced disease severity. Although EAE mice exposed to exercise show reduced levels of activity compared to before EAE onset, TrkB phosphorylation continues to be elevated. These data, along with evidence of increased pro-survival Bcl-2 family member expression in the L4 through L6 region of the spinal cord, suggests that exercise activates intrinsic neuroprotective pathways and may be a meaningful therapeutic strategy for people with MS.

Acknowledgements:

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Figures
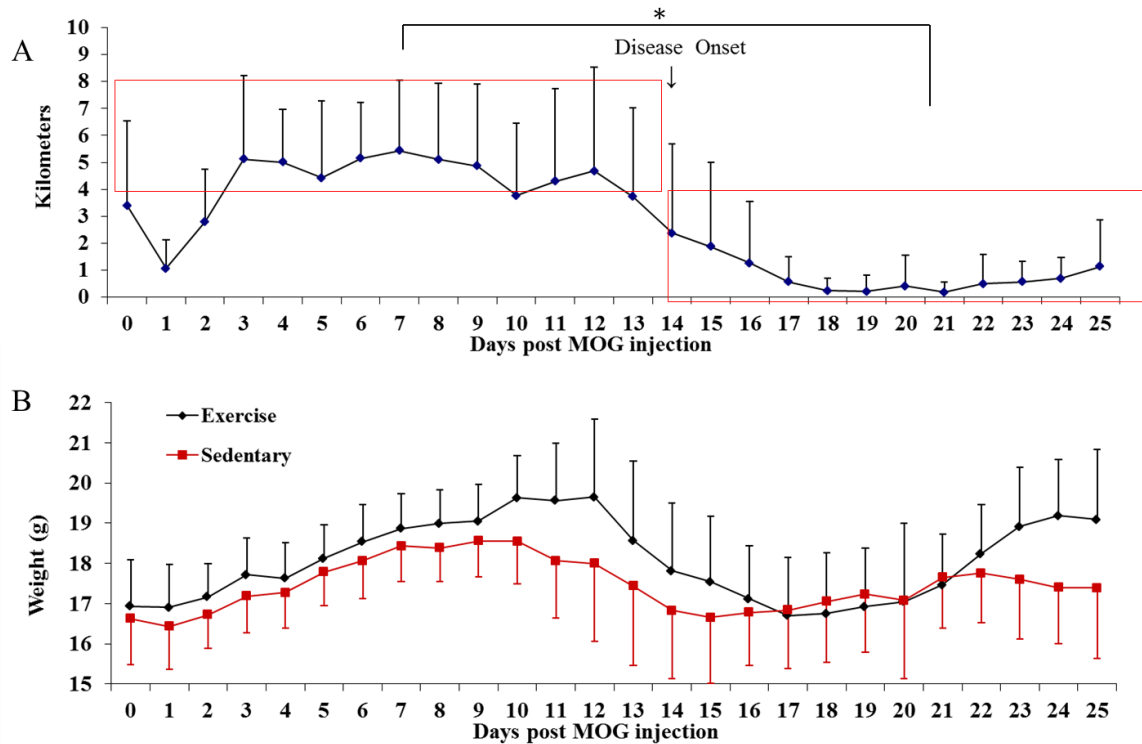
Figure 4.1. Daily distance performed and weight loss associated with EAE onset. Mice were injected with MOG on day 0. (A) Distance was recorded at approximately the same time each morning. Exercise volume was significantly higher prior to disease onset (day 14.25 ± 0.58) (*p<.01; n=8). (B) Weight loss coincides with EAE onset. No significant differences were found between EAE animals exposed to voluntary exercise or sedentary conditions. Data are means $\pm$ SD; n=8/group.
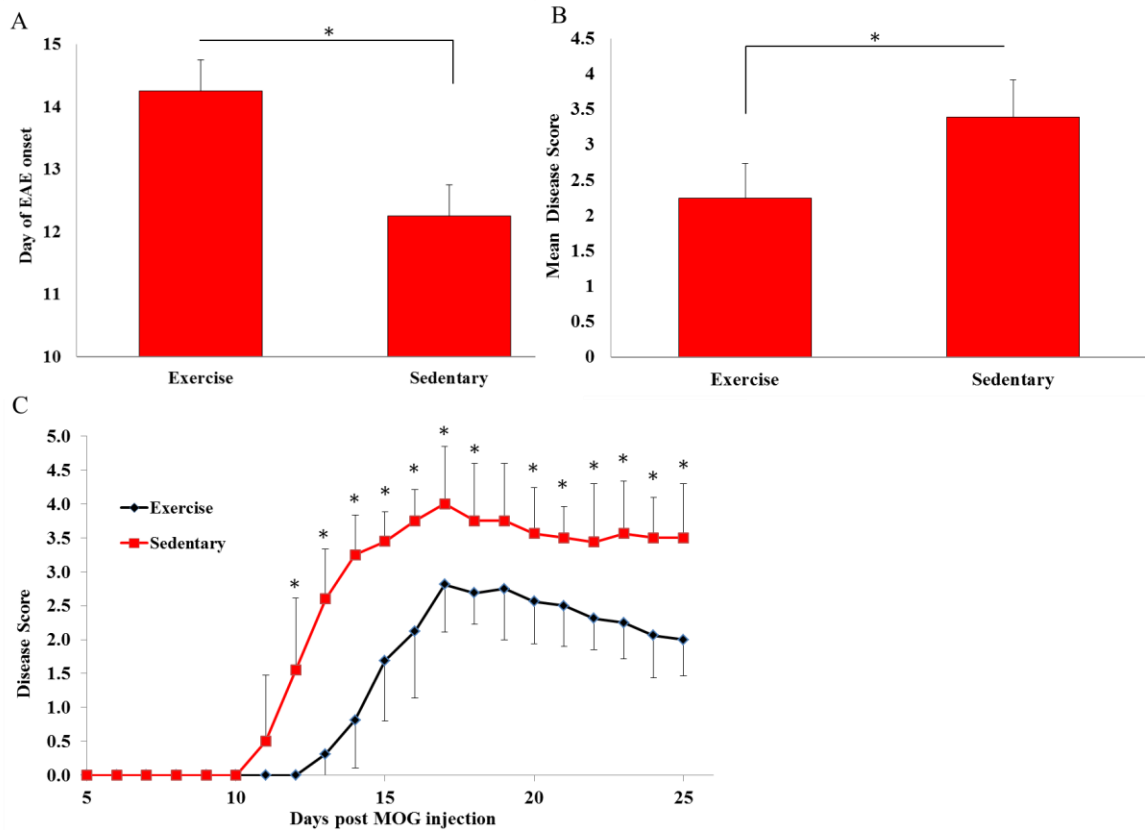
Figure 4.2. Clinical disease course of MOG induced EAE mice. Mice were behaviorally tested daily and scored as described in Methods. (A) The day of disease onset was assessed as the first clinical signs of motor disability, which appeared 2 days earlier in the sedentary group (*p<.01). (B) Differences between mean disease score in exercise and sedentary groups were calculated beginning with the first signs of disease. Data are means ± SD; *p<.01. (C) Daily mean clinical scores are shown for EAE mice exposed to chronic voluntary exercise and sedentary EAE animals. Data are means ± SD; *p<.05; n=8/group.
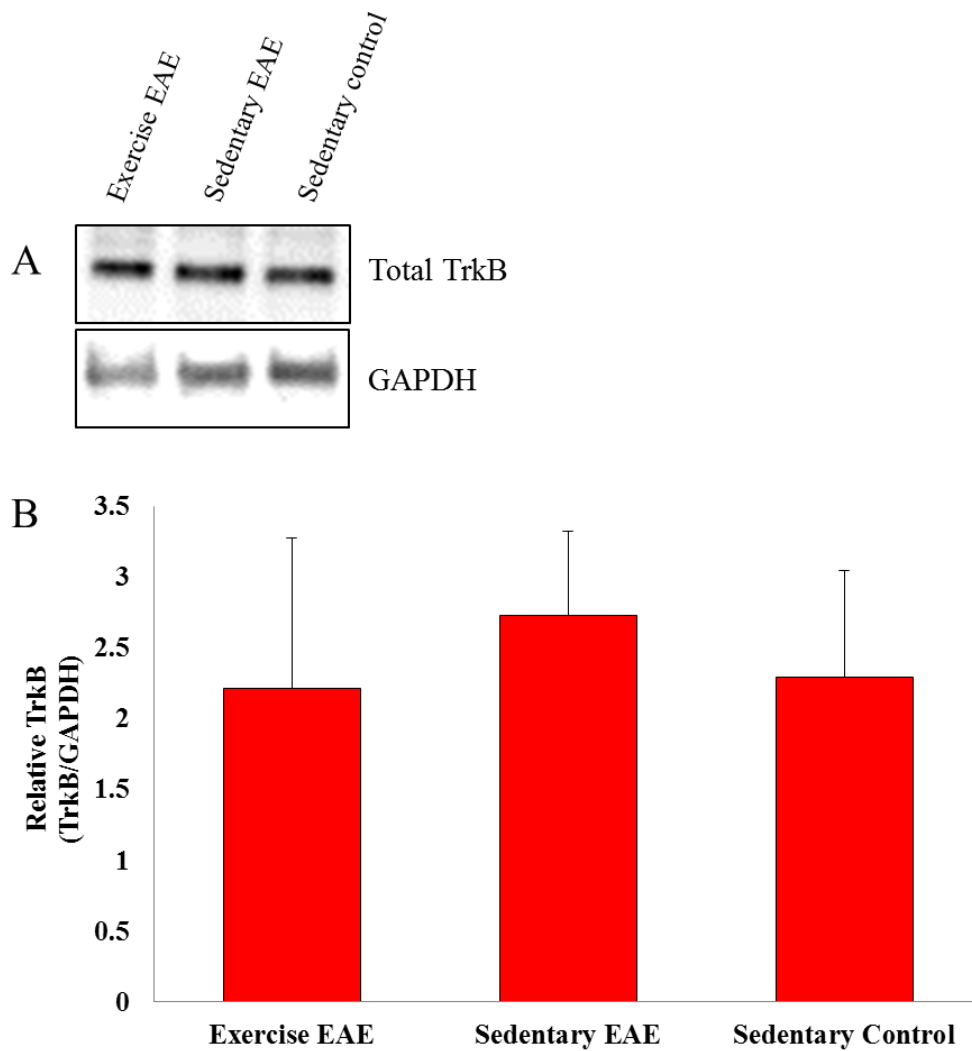
Figure 4.3. (A) Membrane blot of total TrkB expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means ± SD; n=4/group. No significant differences between groups were observed.
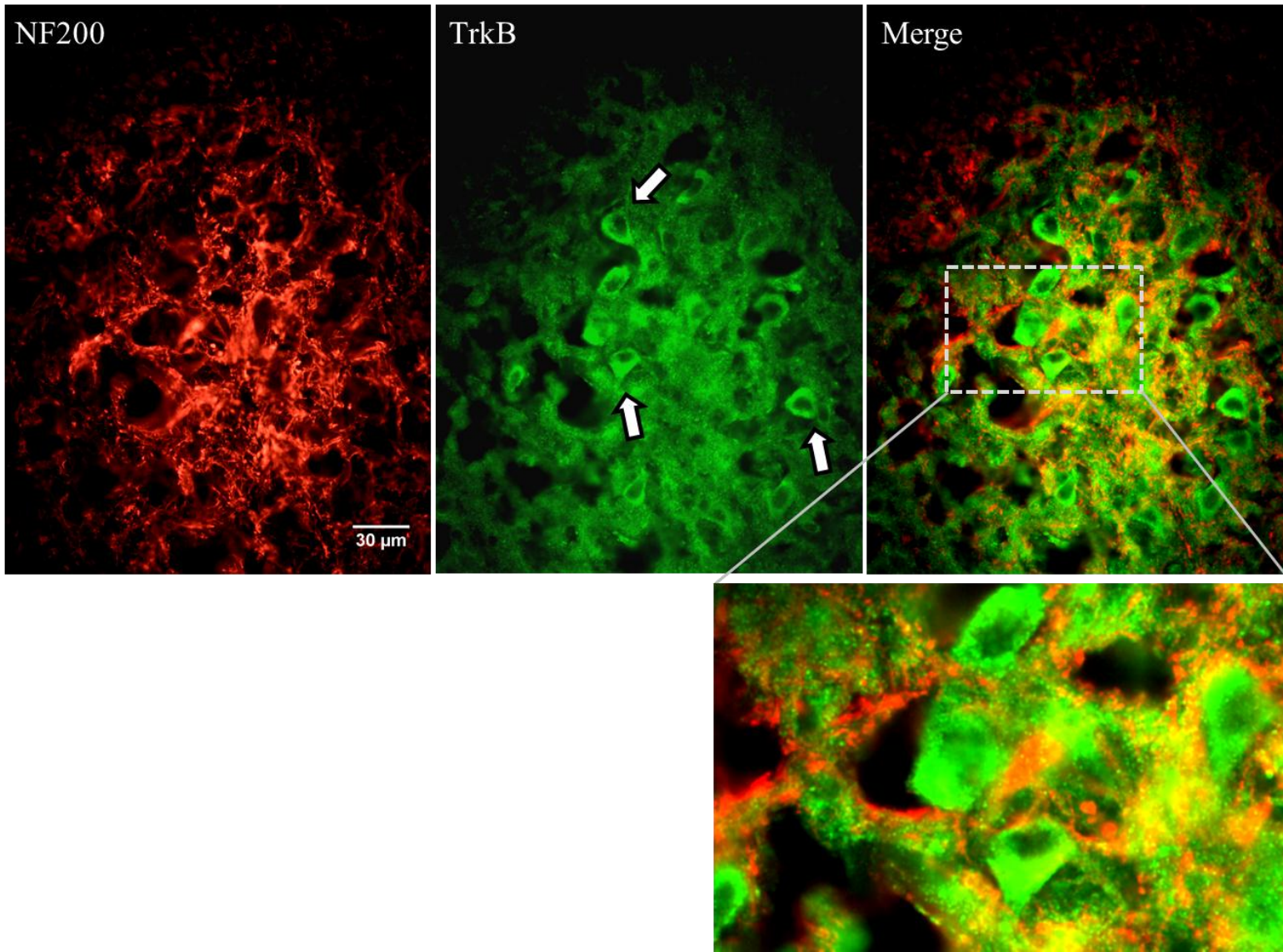
Figure 4.4. Neurofilament heavy chain (NF200) and TrkB immunolabeling in lamina IX of the lumbar spinal cord show that TrkB expression is associated with α-motor neurons and colocalized with axonal processes. Arrows indicate TrkB labeled α-motor neurons.
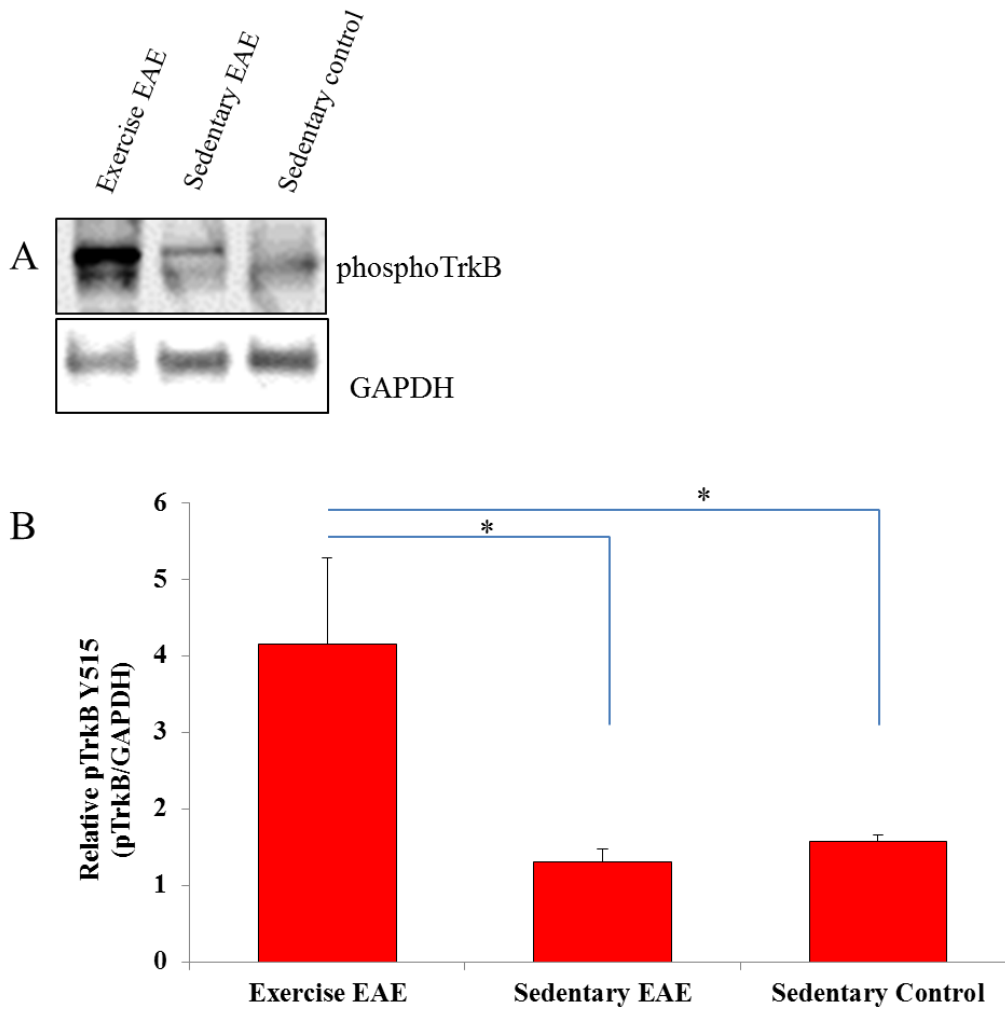
Figure 4.5. (A) Membrane blot of phosphorylated TrkB Y515 expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means $\pm$ SD; *p<.05; n=4/group.
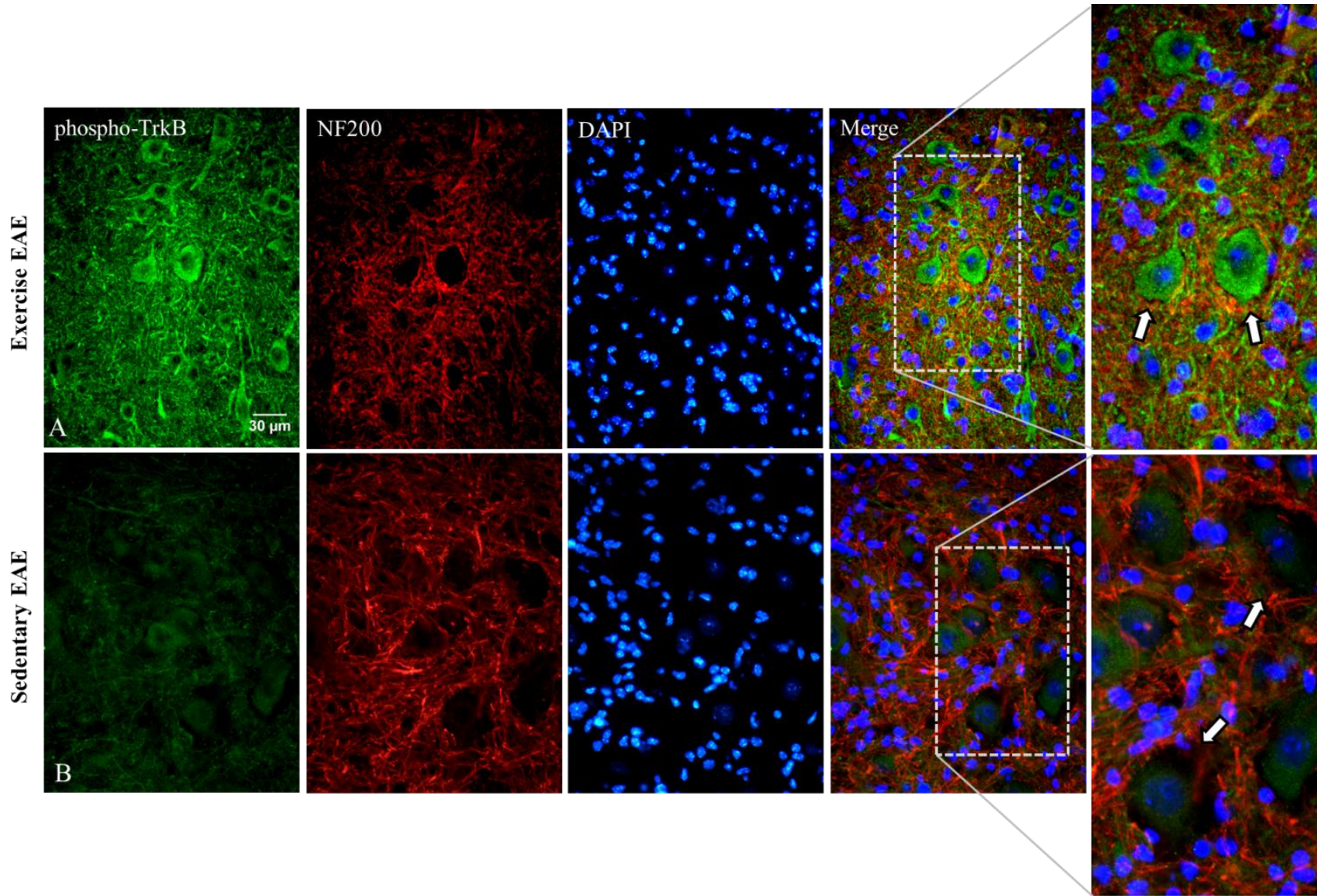
Figure 4.6. Phosphorylated TrkB receptors are heavily phosphorylated in EAE animals exposed to exercise. (A) pTrkB immunolabeling show strong immunoreactivity on α-motor neurons, axonal processes, and other small cells in the lamina IX ventral horn region of L4 through L6 lumbar spinal cord. Merged images show pTrkB immunoreactivity colocalized with neurofilament and associated with smaller cells that appear to be glial cells. (B) EAE animals exposed to sedentary conditions show weak pTrkB immunolabeling, although the distribution throughout the ventral horn appears to be similar. Arrows indicate α-motor neurons.
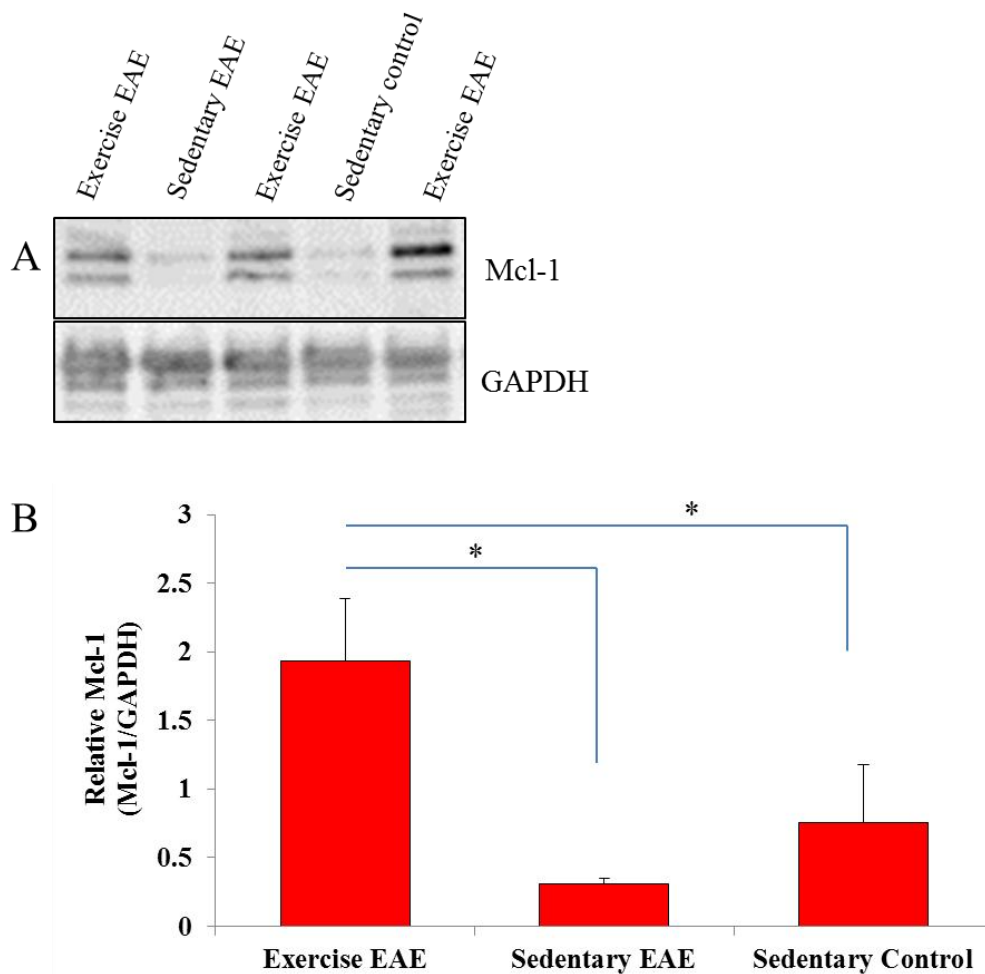
Figure 4.7. (A) Membrane blot of Mcl-1 expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means ± SD; *p<.05; n=4/group
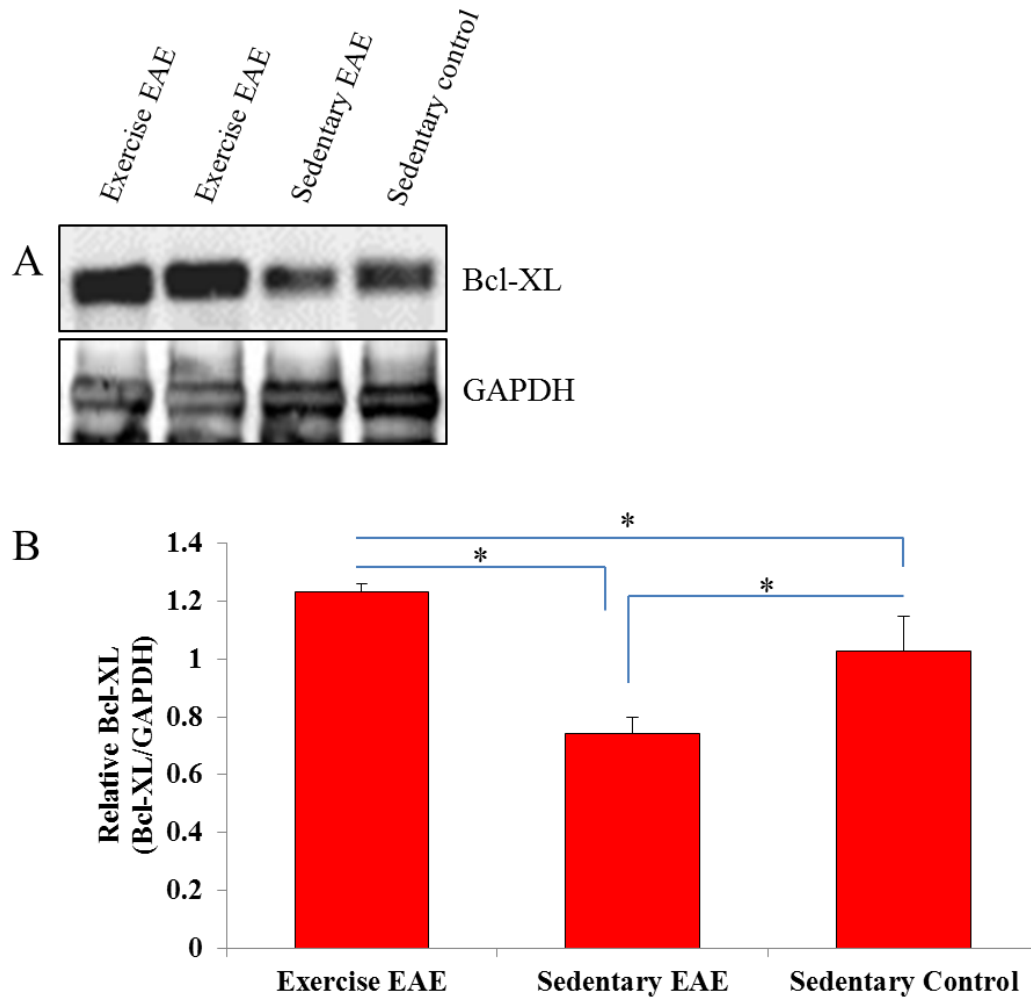
Figure 4.8. (A) Membrane blot of Bcl-XL expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means ± SD; *p<.05; n=4/group.
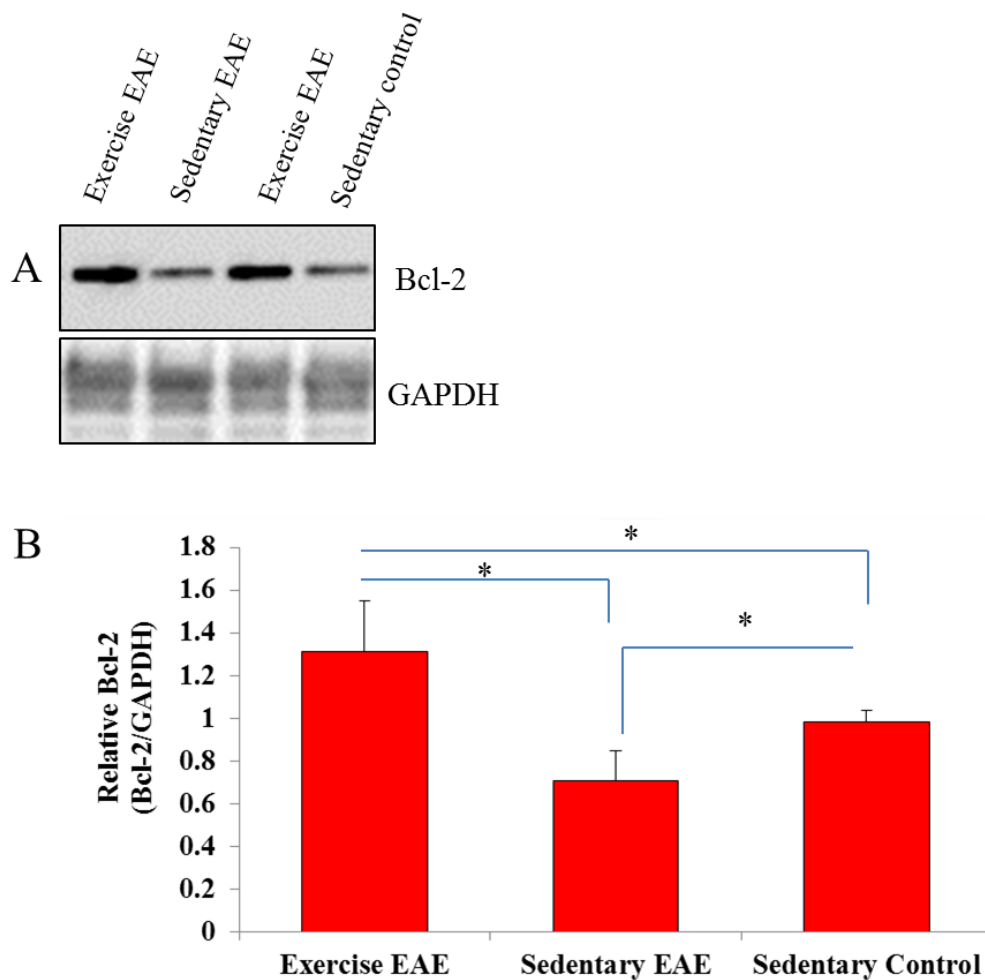
Figure 4.9. (A) Membrane blot of Bcl-2 expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means ± SD; *p<.05; n=4/group.
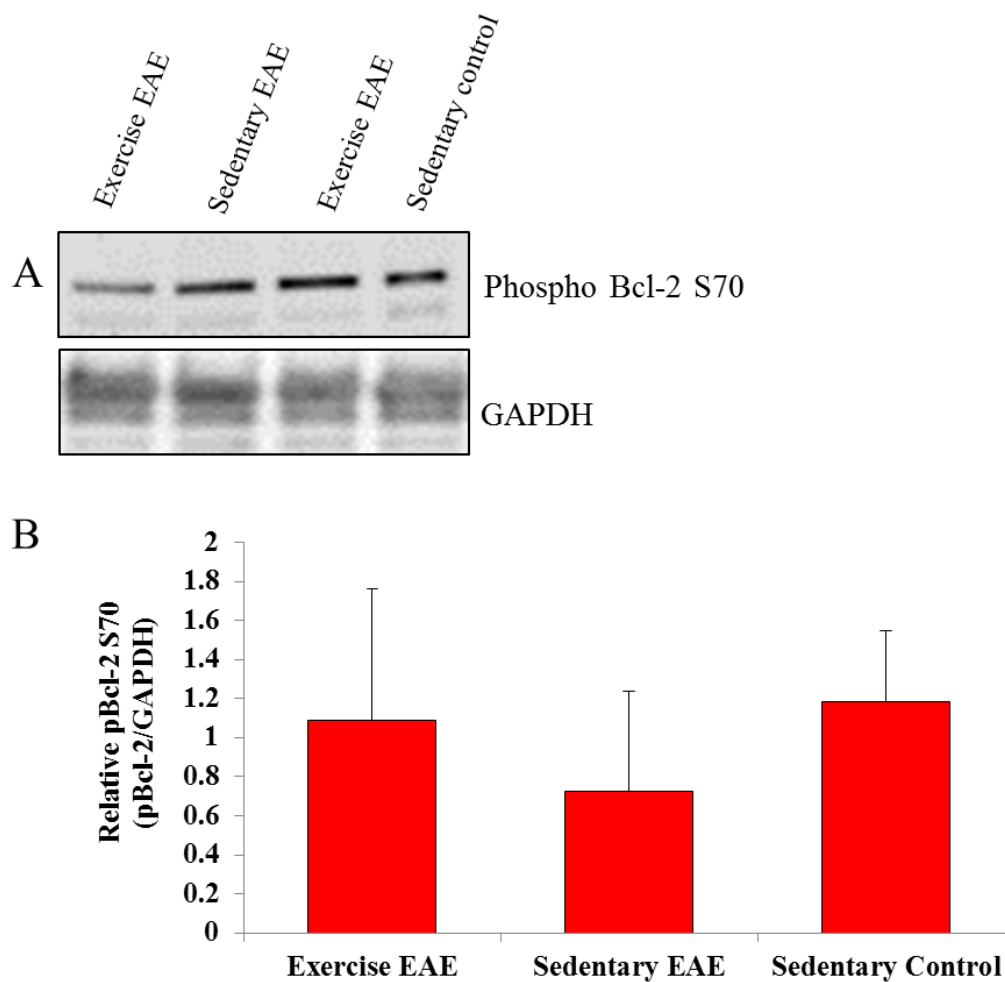
Figure 4.10. (A) Membrane blot of phosphorylated Bcl-2 S70 expression from homogenates of the lumbar spinal cord of exercise EAE, sedentary EAE, and non-EAE sedentary controls. (B) Quantification between groups displayed relative to GADPH controls. Data are means $\pm$ SD; *p<.05; n=4/group.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Multiple sclerosis and its animal model are initiated through myelin associated protein antigen recognition, clonal expansion of autoreactive immune cells in the periphery, and infiltration of those cells into the CNS. The most profound result of our study are data showing that autoimmune cell infiltration into the CNS was greatly reduced when animals were exposed to voluntary exercise during the period of antigen recognition and clonal expansion of autoreactive immune cells. The L5 region of the lumbar spinal cord is the central point where autoimmune T-cells gain entry into the CNS via sensory activation from hindlimb musculature and subsequent alterations in cytokine activation of chemokines (Arima et al., 2012). We found that voluntary exercise was able to attenuate the entry of cells in the ventral white matter tracts, suggesting that although there were obvious increases in sensory activation through chronic exercise, either the gating of cells across the BBB was altered and/or peripheral clonal expansion of autoreactive T-cells was dampened. There is no evidence of the former, which brings about some novel research questions, but the latter has been documented in other animal models of disease. For example, asthma animal models exposed to exercise show altered immune cell infiltration to the respiratory vasculature through increased expression of CD4$^+$ T-regulatory cells in the lymph nodes that provided suppressive effects through the

production of regulatory cytokines and reductions in pro-inflammatory mediators (Lowder et al., 2010).

It is commonly thought that autoimmune diseases are initiated by the breakdown of self-tolerance, suggesting that the recognition of specific antigens by autoreactive T-cells solely contributes to the specificity of autoimmune diseases. Others have recently suggested that there is interaction of the immune system with specific tissue types that leads to autoimmunity (Murakami and Hirano, 2011; Hirano, 2010; Ogura et al., 2008). Tissue activation of CCL20 expression induces autoimmune development through chronic activation of an IL-17 dependent IL-6 signaling amplification loop that is further activated by CD4$^+$ T-cell derived cytokines such as IL-17A, leading to activation of transcription factors that further amplify the positive feedback loop (Murakami et al., 2011). This interaction between cells and tissue is an important area of research. Non-MS healthy controls show a heterogeneous population of anti-myelin T-cell reactivity, yet do not have autoimmune disease. The difference is in the activation of those autoreactive cell populations, where IL-2R$^+$ MBP reactive T-cells are significantly increased in the blood of MS patients when compared with non-MS subjects (Hellings et al., 2001). Tissue-specific autoimmune diseases such as MS may be induced by local tissue type events that cause an accumulation of effector T cells followed by the induction of the IL-6 amplifier in the affected tissue (Murakami and Hirano, 2011). Thus, exercise may alter cytokine profiles and regulatory mediators of the immune response that may interact with the IL-6 amplification loop and reduce tissue specific entry of T-cells across the BBB despite clonal expansion of autoreactive T-cells. This possible mechanisms of cell/tissue interactions and autoreactive activation needs further attention, and may uncover

pharmacological targets that inhibit pathways that otherwise allow immune cells to be activated and gain entry into the CNS.

Another major finding from our experiments in our animal model of MS is that, despite EAE onset and reduced activity during the clinical onset period, animals in the EAE exercise group show a pronounced increase in phosphorylated TrkB receptors over EAE and non-EAE animals exposed to sedentary conditions. It is well recognized that exercise increases BDNF in the CNS and leads to expression of genes involved in plasticity and apoptosis in many cell types including spinal motor neurons, and leads to the activation of cell survival pathways (Keeler et al., 2012; Wu et al., 2011a; Chen and Russo-Neustadt, 2007). Although we did not measure BDNF expression in the lumbar spinal cord, there is a positive relationship between BDNF expression and phosphorylation of TrkB receptors and subsequent downstream signaling (Berchtold et al., 2005). The significant increase in phospho-TrkB receptors in the L4 through L6 region in EAE mice exposed to voluntary exercise may be a more profound than analysis of BDNF levels, since TrkB receptors, when phosphorylated, activate pathways that lead to neuroprotection and expression of genes involved with trophic regulation of the cell. Others have shown that exercise increases phospho-TrkB in the lumbar spinal cord ventral horn neurons and oligodendrocytes within the spinal gray matter after a month of exercise (Skup et al., 2002; Liu et al., 2008; Macias et al., 2007), but this trophic activation has never been reported in the EAE animal model. It comes as a surprise that total TrkB receptors are similar across groups, which suggests that expression of these receptors may not be regulated by exercise or pathology in our model.

In vitro experiments reveal that when cells are deprived of trophic support, they subsequently enter into apoptotic cell death (Kirkland et al., 2010). This *in vivo* model of TrkB trophic deprivation may support the *in vitro* data from our study, as we show that α-motor neurons are reduced by nearly 50% in the sedentary EAE animals compared to EAE mice exposed to chronic voluntary exercise. At this point we cannot conclude that trophic deprivation is the cause of cell death, as cell culture experiments typically deprive cells of all trophic and growth factors to induce apoptosis, while our research only depicts one influential member of the trophic factor family, other ligands were not examined in our experiments. To partly support the hypothesis that BDNF/TrkB is one of the most influential pathways regulated by exercise, gene microarray analysis of the hippocampus shows that during acute and chronic voluntary exercise, there is increased expression of genes involved with synaptic trafficking, signal transduction pathways such as CaMK II, MAPK/ERK I and II, PKC, and transcription regulators such as CREB. BDNF mRNA reveals that this is the only trophic factor consistently upregulated during all timepoints (acute and chronically) and suggests that these signaling pathways are the most responsive to exercise, at least in the hippocampus (Molteni et al., 2002). Further *in vivo* evidence from animal models of neuropathology show that ablation of TrkB receptors or BDNF inhibit exercise-induced neuroprotection and neurogenesis (Liu et al., 2008; Li et al., 2008; Wu et al., 2011a). A future logical step in our experimental process would be to design a construct where TrkB receptors could be knocked-down or ablated specifically in motor neurons to determine if these pathways are responsible for the protective effects of exercise.

Apoptosis serves a crucial role in the development and maintenance of homeostasis in eukaryotic organisms; however, excessive or reduced apoptosis is implicated in many diseases (Thompson, 1995). Analysis of brains from MS autopsy specimens as well as spinal cords from EAE animals show similar apoptotic pathology between MS and EAE, with a 100-fold increase in apoptotic cells, up to 40% of which are myelin producing oligodendrocytes (Dowling et al., 1997). In EAE animal models, apoptosis starts to occur prior to the onset of clinical symptoms, with the highest number of apoptotic cells detected on day one of clinical disease coinciding with increases in activated caspases and pro-apoptotic Bax, with decreases in pro-survival Bcl-2 (Hobom et al., 2004). This increase in pro-apoptotic Bax and a loss of Bcl-2 results in a ratio that favors apoptosis through oligomerization of pro-apoptotic members on the mitochondrial outer membrane and subsequent release of cytochrome-c (Das et al., 2008). Our data show that all three pro-survival members; Bcl-2, Mcl-1, and Bcl-XL, were significantly increased in EAE animals exposed to exercise when compared to sedentary EAE counterparts. This was an interesting finding since the timepoint at which these pro-survival proteins were measured was at least 10 days after the onset of EAE and not at disease onset when apoptosis is most profound. Another key feature of the pro-survival analysis was that in all three pro-survival members, exercise in EAE animals resulted in greater expression above non-EAE sedentary animals, while EAE animals in sedentary conditions displayed a loss of expression when compared to non-EAE animals. It appears that chronic exercise prior to EAE onset, with mild activity throughout the duration of disease, leads to increased and sustained pro-survival member expression, while EAE animals exposed to sedentary conditions have a loss of protein expression.

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Lastly, do these mechanisms from exercise that lead to positive benefits in laboratory animals translate to benefits in the human population? Experimental laboratory mice will run voluntarily on a wheel for many hours at distances near 10 km per day (Lightfoot et al., 2010). Humans with pathology rarely partake in such extreme volumes of exercise, so discovering a volume/intensity threshold for the human population that can elicit these same molecular mechanisms of neuroprotection that is seen in experimental animals is needed. With that said, some researchers use treadmill training to exercise animals and have reported positive effects of exercise on neuroprotection with 30-60 minutes of continuous exercise on most days of the week (Um et al., 2011; Kim et al., 2010a; Kim et al., 2010b). These volumes of exercise can possibly be obtained by people with ambulatory MS. Also, the comparison between animals allowed to exercise and 'sedentary controls' may be confounded by the housing conditions. Animals housed in standard laboratory conditions are typically sedentary, have *ad libitum* access to food, and have little environmental stimuli. These 'sedentary control' animals become overweight, insulin resistant, hypertensive, and are more likely to die prematurely (Martin et al., 2010). Although there are physiological consequences of housing control animals in these conditions, it may actually be a better representation of the physiological conditions of humans. For example, people with MS are more likely to be sedentary and overweight and have increased levels of intramyocellular lipids similar to people with insulin resistance (White et al., unpublished data) and increased cardiovascular risk factors (White et al., 2006).

An intervention that could translate the data gathered from this study using an animal model of MS may be beneficial to individuals with MS, as no current

pharmacological interventions are effective at delaying the progression of disease, and many of these drugs have serious side-effects (Cohen et al., 2010). The data from our animal model study shows that exercise prior to disease onset results in a decrease in autoimmune cell infiltration, which may be translatable to reducing relapse severity in people with MS. As most individuals with MS have relapsing-remitting MS and are ambulatory, then providing a long term chronic aerobic exercise training program may help to attenuate relapse rate and severity. Data from our laboratory reveals that most ambulatory individuals with MS have bilateral differences in motor function and regain some motor abilities with a period of resistance training (White et al., unpublished data). The use of resistance training as a component of the therapeutic protocol is recommended to correct mobility deficits during, or prior to, chronic aerobic training. A statistically powerful sample size and multiple centers should be utilized to perform a long-term intervention centered around chronic exercise in the MS population. This trial should utilize total body progressive resistance training three times per week and prolonged endurance exercise at 50-60% of VO_2 max at a frequency of four to five times per week and be consistently monitored by physical therapists. Outcome variables such as lesion load and grey matter loss will be assessed with MRI, relapse rate and severity will be recorded and monitored by healthcare professionals, and then use a combination of ELISPOT, proliferation assays, and flow cytometry to determine expansion of myelin-derived antigen responding T-cells in the peripheral blood. The goal is to determine if chronic exercise can reduce disease progression, and determine if exercise can alter the autoimmune cell repertoire in people with MS.

In conclusion, chronic voluntary exercise in C57BL/6J mice that is initiated on the day of MOG/adjuvant administration, prior to disease onset, results in attenuated and reduced clinical disease. On day 25 post injection, histology reveals decreases in autoimmune cell infiltration into the L4-L6 region of the lumbar spinal cord. This reduction of cell infiltrates appears to result in preservation of myelination and axons in the ventral white matter tracts. The colocalization experiments show that axons, along with myelin, are degenerated greatly in areas of heavy cellular infiltration, showing that reductions in infiltrates spare anatomy. In normal appearing white matter tracts of the medial ventral regions, axon density is greatly reduced in sedentary EAE animals compared to sedentary EAE animals, and is reflected by behavioral loss of motor abilities in the hindlimbs. Although axons were measured only in areas free of excessive infiltrates, there is reason to believe that lesions upstream of the L4-L6 region have been transected to some degree. In grey matter areas where there are no apparent infiltrates, there is a loss of lamina IX ventral horn α-motor neurons displaying normal morphology in sedentary EAE animals, which suggests that other degenerative processes intrinsic to the CNS likely contribute to the neuropathology of the disease. TrkB activation, which has continually been shown to be neuroprotective *in vitro* and *in vivo,* is highly present in EAE animals exposed to exercise and is all but lost in sedentary EAE animals. Pro-survival members that interact at the outer mitochondrial membrane are also highly expressed in EAE animals exposed to exercise, and are reduced below non-EAE control levels in sedentary EAE animals. These data indicate that chronic voluntary exercise in the EAE model may work in two distinct ways. The first is through mechanisms of

immune system modulation, and second is through activation of intrinsic pathways that favor survival despite neuropathological insult.

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