Exploring Novel Therapies for Motor and Non-Motor Symptoms in a Mouse Model of Multiple Sclerosis.

by

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by neurodegeneration, inflammation and demyelination. Symptoms of multiple sclerosis not only include motor deficits, but also secondary symptoms of pain, depression and anxiety. The purpose of this thesis was firstly to understand the underlying mechanism of these symptoms and secondly to investigate possible treatments. The first half of this thesis investigates the role of several key neurotransmitter systems in the progression and symptomatology of experimental autoimmune encephalomyelitis (EAE), an animal model for MS. The second half of this thesis examines whether exercise can modify the non-motor symptoms associated with EAE.

Experiments in chapter 2 examined the effect of phenelzine (PLZ) in EAE. Daily PLZ treatment, starting seven days after disease induction, delayed EAE onset, reduced disease severity in the chronic phase and was associated with improvements in exploratory behaviour and a novel measure of sickness behaviour. EAE mice treated with daily PLZ showed a recovery in the levels of serotonin (5-HT) and norepinephrine (NE). In particular, EAE mice treated with PLZ showed a normalization of 5-HT levels in the ventral horn of the spinal cord. However, no differences were observed in the amount of T cell infiltration, microglia/macrophage reactivity, demyelination or axonal injury in PLZ-treated spinal cords.

In chapter 3, we conducted experiments in which PLZ treatment only began when mice with EAE exhibited the first clinical signs of the disease, to determine whether PLZ could have beneficial effects in an already established disease state. Using this more clinically relevant treatment approach, we found that PLZ treatment reduced the severity of clinical signs and improved exploratory behaviours for the duration of the experiment in mice with EAE. These

improvements were associated with higher levels of γ-aminobutyric acid (GABA), 5-HT, dopamine (DA) and NE in the brain and spinal cord.

In chapter 4, work was undertaken to assess whether exercise could improve both the motor deficits and secondary symptoms of EAE. Allowing EAE mice 1 hour every day of voluntary running led to a significant delay in the onset of disease symptoms. The development of mechanical allodynia was assessed using Von Frey hairs and indicated that wheel running had a modest positive effect on the pain hypersensitivity associated with EAE. These behavioural changes were associated with decreased CNS inflammation, reduced oxidative stress and less mitochondrial protein expression. Unlike the case in chapters 2 and 3, voluntary wheel running led to only minor changes in neurotransmitter levels.

The work done in chapter 5 further investigates the mitochondrial changes that occur during early EAE by examining the proteins involved in mitochondrial fission, fusion and biogenesis. EAE animals had increased levels of both the outer mitochondrial membrane protein Tom20 and the complex IV protein (COX IV) within the dorsal horn of the spinal cord compared to control mice. This increase was associated with an increase in the mitochondrial fission mediator, Drp1, a decrease in phosphorylated Drp1 at ser616, a decrease the fusion protein mitofusin 2 (MFN2), and a decrease in the expression of peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α).

The work in this thesis demonstrated the therapeutic potential of the monoamine oxidase (MAO) inhibitor PLZ and of daily exercise in MS. This study led to the identification of the role of several key neurotransmitters and also highlights mitochondrial dysfunction in the secondary symptoms of the disease. Further research into these mechanisms may lead to the development of novel therapies for MS.

Preface

This thesis is the original work of C. Benson. The projects and protocols used in this thesis have been approved by the University of Alberta animal care and use committee.

Chapter 1 of thesis is an expanded version of the paper published by Benson C and Kerr BJ (2014) Pain and cognition in multiple sclerosis. *Curr Top Behav Neurosci* 20:201-15. C. Benson was involved in concept formation and manuscript composition and editing. B.J. Kerr was involved in manuscript editing and was the supervisory author.

Chapter 2 of this thesis has been published as Musgrave T[1], Benson C[1], Wong G, Browne I, Tenorio G, Rauw G, Baker GB, Kerr BJ., (2011). The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE). *Brain Behav Immun* 25:1677-1688. T. Musgrave and C. Benson contributed equally to this work. T Musgrave was responsible for data collection and analysis of Figures 2.1 and 2.2 with assistance from C. Benson. C. Benson was responsible for data collection, analysis and preparation of figures 2.3-2.7 with assistance from G. Wong on figures 2.3 and 2.6. G. Rauw, G. Tenorio and I. Browne provided technical assistance. C. Benson, T. Musgrave and B.J. Kerr were involved in concept formation and manuscript composition and editing. G.B. Baker and B.J. Kerr were the supervisory authors.

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“Every day is a new day. It is better to be lucky. But I would rather be exact. Then when luck comes you are ready.”
— Ernest Hemingway, The Old Man and the Sea

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List of Abbreviations

5-HIAA 5-hydroxyindole-3-acetic acid
5-HT 5-Hydroxytryptamine, serotonin
APC Professional antigen presenting cells
BDNF Brain-derived neurotrophic factor
CCI Chronic Constriction Injury
CFA Complete Freund's Adjuvant
CNS Central nervous system
COXIV Complex IV
CSF Cerebrospinal fluid
DA Dopamine
DMF Dimethyl fumarate (DMF),
DMT Disease modifying therapies
Drp1 Dynamin-related protein 1
EAAT-2 Excitatory amino acid transporter-2
EAE Experimental autoimmune encephalomyelitis
EC Eriochrome Cyanine
ETC Electron transport chain
FIS1 Mitochondrial Fission Protein 1
GABA Gamma-Aminobutyric acid
GABA-T GABA Transaminase
GAD65 Glutamic acid decarboxylase
GAT-2 GABA transporter type 2
GFAP Glial Fibrillary Acidic Protein
GLY Glycine
GM-CSF Granulocyte-macrophage colony-stimulating factor
GSH Glutathione
GSSG Glutathione disulfide
GWAS Genome wide association studies
H_2O_2 Hydrogen Peroxide
HLA Human leukocyte antigen
HPA Hypothalamo-pituitary- axis
HPLC High Performance Liquid Chromatography
IBA1 Ionized calcium-binding adapter molecule 1

IBC N-isobutyryl-L-cysteine
IFNγ Interferon-γ
IL Interleukin
iNOS Inducible nitric oxide synthases
IP Intraperitoneal
JCV John Cunningham Virus
LC Locus coeruleus
LIF leukemia inhibitory factor
LPS Lipopolysaccharides
MAIT Mucosal-associated invariant T cells
MAO Monoamine oxidase
MBP Myelin basic protein
MFN2 Mitofusin 2
MHC major histocompatibility complex
MHV Mouse hepatitis virus
MOG Myelin oligodendrocyte glycoprotein
MS Multiple sclerosis
mtDNA Mitochondrial DNA
NAA N-Acetylaspartate
NADPH Reduced Nicotinamide Adenine Dinucleotide Phosphate
NAWM Normal appearing white matter
NE Norepinephrine/Noradrenalin
NK Natural killer cells
OPA *o*-phthaldialdehyde (OPA)
PB Phosphate buffer
PBMC Peripheral Blood Mononuclear Cell
PEH phenylethylidenehydrazine
PGC-1α Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PLP Proteolipid protein
PLZ Phenelzine
pNR1 Phosphorylated NMDA receptor
PNS Peripheral nervous system
PPMS Primary progressive multiple sclerosis
PML Progressive multifocal leukoencephalopathy
ROS Reactive oxygen species

RRMS Relapsing remitting multiple sclerosis
SERT Serotonin transporter
SOD Superoxide Dismutase
SPMS Secondary progressive multiple sclerosis
TCR T cell receptor
THF Tetrahydrofuran
TMEV Theiler's murine encephalomyelitis virus
TN Trigeminal neuralgia
Tom20 Translocase of Outer Membrane 20
TRPV1 Transient Receptor Potential Cation Channel Subfamily V Member 1

Chapter 1

General Introduction

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a degenerative disease in which the myelin of the central nervous system (CNS) is attacked by autoimmune processes. Progressive demyelination leads to the loss of proper neuronal, signaling causing weakness and, ultimately paralysis. Demyelinating lesions can occur throughout the white matter in the brain and spinal cord, leading to a variety of symptoms that are hallmarks of the disease. The initial symptoms of MS include muscle weakness, optic neuritis (due to demyelination of the optic nerve), reduced dexterity and ataxia (Hauser, 2006). In addition to these classic motor disabilities, cognitive impairments, increased pain and depression are also common with disease progression (Grasso et al., 2008; Langdon, 2011).

There are 4 established presentations of MS: relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS) and progressive relapsing. The majority of patients present with RRMS. RRMS is characterized by acute periods of disability followed by periods of remission. Regardless of treatment, a significant number of patients with RRMS will develop SPMS, which is characterized by persistent disability. A subset of people will present with PPMS, where the disease progresses quickly and there are no periods of remission (Compston and Coles, 2002).

1.2 BRIEF EPIDEMIOLOGY OF MS

MS is more common in Canada, Europe, the United States, New Zealand, and Australia compared to other countries around the world. This exemplifies the observation that there is a greater MS incidence with increasing distance from the equator (Kurtzke, 1995). However, more recent studies have shown that prevalence of MS across the United States does not correlate with

latitude and may indicate a changing distribution of the disease (Koch-Henriksen and Sorensen, 2011). Across all countries, women are significantly more likely to be affected by MS; the current estimate of the female to male ratio is 2.5:1 (Koch-Henriksen and Sorensen, 2010). In addition, people of northern European descent have a particularly high rate of MS; however recent studies suggest that the risk of MS is increasing in other populations (Wallin et al., 2012). Although the cause of MS is unknown, environmental factors have been associated with an enhanced risk of MS. The most common environment risk factors include vitamin D levels, obesity in early life, Epstein-Barr virus (EBV) infection, and cigarette smoking. A combination of these risk factors likely contributes to the unequal distribution of MS across the world and amongst populations.

1.2.1 MS Risk Factors

Low vitamin D levels have been recognized as a risk factor for MS. Using pre-onset blood samples from MS patients and matched controls, researchers found that having a higher serum concentration of 25-hydroxyvitamin D was associated with a lower risk of MS (Munger et al., 2006). Diet and sun exposure are sources of vitamin D and both have been correlated with MS risk, yet the relative importance of these two sources remains unclear (Bjornevik et al., 2014; Kampman et al., 2007). Gene variations that lead to lower 25-hydroxyvitamin D production have also been associated with an increased risk of MS (Wang et al., 2010). The mechanistic role of vitamin D in MS is still being explored, but there is evidence supporting an immune modulatory effect of vitamin D (Hayes et al., 2015). Another modifiable risk factor for MS is obesity. Obesity early in life has been associated with an increased risk of MS in both males and females (Hedstrom et al., 2012). The mechanism underlying this effect is unknown, but could involve the lower vitamin D levels in obese individuals or the effect of adipose tissue on immune regulation

(Pereira-Santos et al., 2015). The effect of EBV infection on immune regulation also contributes to an increased risk of MS. Circulating antibodies specific for the EBV is a strong predictor of MS risk (Ascherio et al., 2001). Although MS in the absence of EBV sero-positivity is rare, the majority of the general population also carries antibodies for EBV (Ascherio et al., 2001), thus suggesting that EBV needs to occur in combination with other factors to generate MS disease. The mechanism by which EBV contributes to MS risk is unknown. However, the inflammatory response to EBV may promote cross reactive T-cells and, in some individuals, this may lead to autoimmune disease development (Lang et al., 2002). Another significant environmental risk factor for MS is cigarette smoking. The association between an increased risk of MS and smoking has been observed throughout the world (Riise et al., 2003). However, smoking may have a greater impact on the MS risk of males compared to females (Ramagopalan et al., 2013). The mechanism by which cigarettes promote MS risk is unknown. However, smoking is known to enhance the risk of other autoimmune diseases, which suggests a general effect of smoking on immune regulation (Costenbader and Karlson, 2006). The factors discussed above are modifiable and therefore offer a straightforward way to reduce an individual's risk of MS.

1.3 APPROVED THERAPIES FOR MS

In Europe and the United States there are currently 9 classes of disease modifying therapies (DMT) approved for the treatment of MS. These include type 1 interferons. glatiramer acetate, natalizumab, fingolimod, teriflunomide, dimethyl fumarate (DMF), alemtuzumab, mitoxantrone and daclizumab (Wingerchuk and Weinshenker, 2016). These therapies have varied immunological mechanisms, but have all been shown to reduce either the annualized relapse rate, lesion number, or overall disability. Below is brief description of the mechanisms and any major adverse events associated with each of the currently available treatments for MS.

1.3.1 Interferon-beta

Interferon-beta was first approved in 1993, but interestingly was never underwent pre-clinical testing in EAE (Jacobs et al., 1982). Interferon-beta is an self-injection taken every 2 days or every 2 weeks in the case of the newer formulations (Calabresi et al., 2014). Compared to placebo, interferon-beta reduces relapse rates by 30% (Jacobs et al., 1996). Multiple mechanisms have been found to underlie the positive effect of interferon-beta. For example, interferon-beta treatment has been shown to promote blood-brain barrier stability, mediate a shift in inflammatory response from a pro-inflammatory Th1 response to an anti-inflammatory Th2 response, and decrease antigen presentation by glial cells (Jiang et al., 1995; Stuve et al., 1996). Importantly, interferon-beta is well tolerated by patients and is not associated with severe immunesuppression, a common worry with these types of treatments. Although there are few adverse side effects associated with this treatment, newer MS therapies have proven to be more effective. In addition, the frequency of the injections can be a barrier to the patients' adherence to their treatment schedules. (Cohen et al., 2015; Miller et al., 2003).

1.3.2 Glatiramer acetate

Glatiramer acetate is an analogue of myelin basic protein and is a mixture of polypeptides with four amino acids in random order (Weber et al., 2007). In the periphery, glatiramer acetate binds to the major histocompatibility complex (MHC), which interferes with proper antigen presentation. This then promotes a Th2 response and an increase in regulatory T-cells, both of which are thought to have beneficial effects on disease course (Blanchette and Neuhaus, 2008). Furthermore, glatiramer acetate treatment is associated with increased brain-derived neurotrophic factor, which may promote the survival of neurons and glial cells (Sarchielli et al., 2007b).

Glatiramer acetate is also well tolerated and has an efficacy similar to interferon-beta (La Mantia et al., 2014).

1.3.3 Natalizumab

Natalizumab is a humanized monoclonal antibody targeted to very late antigen-4. This antigen is expressed on the surface of monocytes and lymphocytes and is involved in the transmigration of these cells into tissue (Engelhardt et al., 1998). Natalizumab acts by blocking the binding of very late antigen-4 to ligand vascular cell adhesion molecule-1, thereby limiting the ability of immune cells to cross into the CNS (Ransohoff, 2007). Natalizumab has been shown to be more effective at reducing relapse rates than both interferon-beta and glatiramer acetate (Miller et al., 2003). Although natalizumab may be more effective at treating MS, it is associated with a significant risk of John Cunningham Virus (JCV)-associated progressive multifocal leukoencephalopathy (PML) (Borchardt and Berger, 2016). PML is an often fatal demyelinating disorder associated with JCV infection of glial cells within the brain (Tan and Koralnik, 2010). It is thought that natalizumab suppresses the normal immune monitoring of the brain, allowing PML to occur unchecked (Aly et al., 2011). The incidence of PML in MS patients taking natalizumab has been assessed at 2.1 cases per 1000; however in this may in fact be an underestimation. (Bloomgren et al., 2012; Borchardt and Berger, 2016). This risk can be stratified based on three risk factors: prior immunosuppressive treatment, previous JCV infections, and a use of natalizumab greater than 24 months. A patient with none of these factors has a risk of 0.001%, while having all three factors is linked to a risk of 1.1% (Bloomgren et al., 2012). However, a recent study that used a Kaplan-Meier statistical method found that the realistic risk of PML was 2 to 9 times greater than previously reported (Borchardt and Berger, 2016). This paper also suggested using the markers

L-selectin and CSF lipid-specific IgM bands as a further way to stratify the risk of natalizumab-associated PML (Borchardt and Berger, 2016)

1.3.4 Fingolimod

Fingolimod was approved in 2012 and was the first oral DMT for MS. This treatment acts as a functional antagonist of the sphingosine-1-phosphate receptors. This receptor is involved in the exit of CCR7-expressing lymphocytes from lymph nodes. Fingolimod treatment down-regulates sphingosine-1-phosphate receptors, thereby confining CCR7 positive cells to the lymphoid tissue and preventing their migration into the CNS (Chun and Hartung, 2010; Francis et al., 2014). . However, fingolimod treatment is associated with an increased risk of infection. Varicella zoster virus, herpes simplex virus and cryptococcal infections have all been reported with fingolimod use (Pfender et al., 2015). In addition, several cases of PML have been observed in patients receiving fingolimod treatment (Faulkner, 2015).

1.3.5 Teriflunomide

Teriflunomide is a daily oral therapy and is an inhibitor of the mitochondrial enzyme dihydroorotate dehydrogenase. Blocking this enzyme inhibits de-novo pyrimidine synthesis, which results in a reduction in T-cell and B-cell proliferation (Bar-Or et al., 2014). Teriflunomide does not cause severe immune suppression, however it has a similar effectiveness as interferon-beta and glatiramer acetate (O'Connor et al., 2011).

1.3.6 Dimethyl fumarate (DMF)

DMF is derived from fumaric acid, and in Germany has been used in a derivative form to treat psoriasis (Reich et al., 2009). The exact mechanism underlying the beneficial effects of DMF is still unclear. However, DMF has been shown to have immune modulatory properties. For

example, DMF can induce a shift from Th1 to Th2 immunity and can promote anti-inflammatory dendritic cells (de Jong et al., 1996; Ghoreschi et al., 2011). Interestingly, DMF is also able to induce an anti-oxidative stress response through the translocation of the transcription factor nuclear factor E2-related factor 2 (Nrf2) into the nucleus (Linker et al., 2011). Once in the nucleus, Nrf2 promotes the transcription of several antioxidant genes, which may lead to the protection of neurons and glia in MS. DMF treatment can also lead to significant decreases in lymphocyte number. Unfortunately, cases of PML have occurred with prolonged treatment (Ermis et al., 2013; Longbrake et al., 2015).

1.3.7 Alemtuzumab

Alemtuzumab is a humanized monoclonal antibody specific for CD52. CD52 is a surface marker on lymphocytes and, to a lower extent, monocyte-derived cells. Treatment with alemtuzumab causes a large reduction in $CD4^+$ T-cells and B-cells. This loss is likely a result of antibody-dependent cell-driven or complement-dependent cytotoxicity (Bologna et al., 2011; Hu et al., 2009). Lymphocyte recovery usually begins 6 months to 1 year after treatment, but despite this, some patients still see benefits from the treatment for multiple years (Kousin-Ezewu et al., 2014). Uniquely, alemtuzumab treatment is associated with a significant risk of acquired autoimmune disease. In a 5-year follow-up study, approximately 30% of MS patients treated with alemtuzumab experienced an autoimmune attack of the thyroid (Daniels et al., 2014). These autoimmune conditions are thought to occur from an imbalance in the types of lymphocytes that repopulate after treatment with alemtuzumab (Jones et al., 2013).

1.3.8 Daclizumab

Daclizumab is a humanized monoclonal antibody targeting CD25, which is the α-subunit of the interleukin-2 receptor. CD25 is expressed on activated T-cells, and disruption of CD25 on these cells by daclizumab decreases T-cell expansion and significantly increases regulatory CD56 natural killer cells (Wiendl and Gross, 2013). In MS patients, daclizumab treatment can correct the impaired signalling between CD56 natural killer cells and T-cells, leading to the lysis of activated $CD4^+$ T-cells (Gross et al., 2016). Daclizumab treatment showed similar efficacy as interferon beta; however daclizumab was associated with a greater number of adverse events (Kappos et al., 2015).

1.3.9 Mitoxantrone

Lastly, the anti-cancer drug mitoxantrone has been approved for use in aggressive cases of MS. Unfortunately, the use of mitoxantrone in MS is limited due to the risk of life threatening cardiotoxicity and development of acute leukemia associated with long term use (Cocco and Marrosu, 2014). Mitoxantrone causes widespread immune suppression of proliferating T-cells, B-cells and macrophages (Neuhaus et al., 2005).

1.4 ANIMAL MODELS FOR MS

There are a variety of animal models used to replicate different aspects of MS pathophysiology and the behavioral deficits associated with the disease. The animal model that is most widely used is experimental autoimmune encephalomyelitis (EAE). This model has contributed significantly to our understanding of MS pathophysiology (Ransohoff, 2012). EAE was first discovered in 1925 during experiments exploring the neurological side effects of the rabies vaccine. At the time, the rabies vaccine was generated using neural tissue, which occasionally

led to pathologies not associated with rabies. Experiments with non-human primates determined that the components of the vaccine that included neural tissue induced acute CNS inflammation (Ransohoff, 2012). This CNS inflammation generated a disorder that was characterized by impaired gait and paralysis, which was associated with an inflammatory reaction and spinal demyelination, hallmark symptoms of EAE (Rivers et al., 1933).

Significant improvements in the EAE model(s) have occurred over the past 90 years, with multiple antigenic peptides and strains of animals being used to induce the disease. The most common peptides used are components of myelin: myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG). Currently the majority of studies induce EAE in the C57BL/6 strain of mice using the antigenic MOG_{35-55} peptide fragment that is emulsified in Complete Freund's Adjuvant (CFA). Mice are additionally treated with pertussis toxin on the day of induction and again 2 days later. Classically, this protocol generates a monophasic disease progression with little to no relapses. However, by varying the concentration of the MOG peptide and the CFA in the emulsion, a relapsing and remitting disease phenotype can be produced (Berard et al., 2010).

In addition, MS like symptoms can be generated using T cell receptor (TCR) transgenic mouse models. The T-cells within these animals do not undergo TCR recombination and therefore create a large population of T-cells with a single defined epitope. An advantage of these transgenic models is that they can be designed to more closely resemble MS. Specifically, these models develop EAE spontaneously and can be generated such that the infiltrating immune cells can be CD4 and CD8 T-cells and B-cells (Bettelli et al., 2006; Bettelli et al., 2003).

Viral infections can also induce CNS demyelination in mice and can be used to study MS. The most common virus used is the picornavirus, Theiler's murine encephalomyelitis virus

(TMEV). These viruses directly infect neurons, causing neurodegeneration and demyelination. Moreover, unlike EAE where inflammatory demyelination precedes axonal damage, during TMEV infection, demyelination is secondary to axonal injury (Tsunoda et al., 2003). Therefore, TMEV models aspects of the "inside-out: theory of MS. This theory suggests that primary axonal injury leads to the recruitment of T-cells into the CNS, which leads to subsequent demyelination (Amor et al., 2010).

1.5 IMMUNE CELLS AND MS

1.5.1 T-cells in MS

T-cells play a significant role in the pathology of the disease. T-cells can be detected within the CNS early in MS disease progression and are thought to be the primary auto reactive component of MS. The importance of T-cells in MS is highlighted in the results of genome wide association studies (GWAS) that have identified specific human leukocyte antigen (HLA) class II gene variants as contributing significant risk for developing MS (Sawcer et al., 2011). These genes direct which antigens are displayed on professional antigen presenting cells (APC) and thereby control the specificity of the T-cell population. Given that demyelination is a hallmark feature of MS, researchers have looked for myelin reactive T-cells in the blood of MS patients. Although myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) specific-cells can be found in the blood of MS patients, they can also be found circulating in healthy adults, muddying the significance of this line of research (Pette et al., 1990). Despite this, several effective therapies such as Natalizumab and Fingolimod have been developed that disrupt T-cell function in MS, suggesting that these immune cells contribute to MS disease (Haghikia et al., 2013).

1.5.2 Th1 and Th17

EAE disease progression is characterized and driven primarily by $CD4^+$ T-cells (Robinson et al., 2014). In general, EAE begins with peripheral immune activation to a myelin epitope and subsequent CNS infiltration of $CD4^+$ T-cells. The migration of T-cells into the CNS and prior microglial activation causes the infiltration of peripheral monocytes. These blood-borne monocytes likely cause significant CNS damage, which correlates with disease severity (Ajami et al., 2011). In EAE, the $CD4^+$ T-cells can be subgrouped into Th1 and Th17. These subsets are induced by the CNS micro environment and are defined by the cytokines they secrete: in general Th1 cells produced IFN-γ and TNF-α and Th17 cells produce Il-17, Il-21 and Il-22 (Legroux and Arbour, 2015). Th1 and Th17 cells are considered pro-inflammatory, however their exact role in EAE and MS is complex. Adoptive transfer of either myelin reactive Th1 or Th17 cells into naive mice is sufficient to cause EAE disease (Fletcher et al., 2010; Langrish et al., 2005). However, the induction of EAE in mice lacking either soluble IFN-γ or IFN-γ receptor showed greater disease severity, suggesting that IFN-γ producing cells are not critical for disease pathogenesis (Ferber et al., 1996) Similarly, mice deficient in IL-17, IL-21 or IL-22 were still fully susceptible to EAE (Haak et al., 2009; Kreymborg et al., 2007; Sonderegger et al., 2008). Recently, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to be released by both Th1 and Th17 cells and mice lacking GM-CSF are resistant to EAE (Codarri et al., 2011). This result indicates that GM-CSF may be a required for EAE autoimmunity.

1.5.2 $CD8^+$ T-cells

Within an active MS lesion the composition of lymphocytes is dominated by $CD8^+$ T-cells (Mars et al., 2011). Although, $CD8^+$ T-cells cannot directly respond to class II antigen presentation,

they likely have a key role in MS disease progression. Both active and memory phenotypes of $CD8^+$ T-cells have been observed with the CNS of MS patients (Zang et al., 2004). In both SPMS and PPMS and to a lesser amount RRMS, $CD8^+$ T-cells can be found diffusely in normal appearing white matter (NAWM) (Kutzelnigg et al., 2005). These $CD8^+$ T-cell infiltrates were also associated with reactive microglial. In addition, $CD8^+$ T-cells can be detected in close proximity to oligodendrocyte in MS brain samples and *in vitro* studies indicate that $CD8^+$T-cells are capable of lysing CNS cells (Jurewicz et al., 1998; Lassmann et al., 2007). Furthermore, these cells can support neuroinflammation by secreting TNF-α and IFN-γ and a subset of $CD8^+$ cells called mucosal-associated invariant T cells (MAIT) can produce Il-17 (Crawford et al., 2004; Willing et al., 2014).

1.5.3 Macrophages/Microglia in MS

Macrophages and microglia have been extensively studied for their role in MS and EAE. Macrophages exist on a continuum of polarization between pro-inflammatory and anti-inflammatory states driven by signals in the microenvironment (Rawji and Yong, 2013). Shifting the balance towards an anti-inflammatory state could possibly be an effective treatment strategy in MS. This strategy could be used to block the entry of T-cells into the CNS by targeting the macrophage populations that reside near key interfaces between the CNS and peripheral blood. The choroid plexus, vasculature and the meninges have been identified as critical points of entry for T-cells into the CNS.

1.5.4 Perivascular Macrophages

Cells that infiltrate into the CNS via the vasculature must pass first through the vessel endothelium, then the perivascular space and finally the astrocytic glia limitans (Varatharaj and

Galea, 2016). In MS and EAE, macrophages and dendritic cells within the perivascular space are capable of influencing disease progression. The number of $CD163^+$ perivascular macrophages increases in MS and EAE (Bauer et al., 1995; Fabriek et al., 2005). Interestingly, this increase can be detected prior to the infiltration of peripheral cells into the CNS and the onset of clinical signs in EAE. In MS and EAE, perivascular macrophages express high levels major histocompatibility complex (MHC) II and adhesion molecules, such as ICAM-1, VCAM-1 and CCL2 (Hofmann et al., 2002). This suggests that these cells may promote the activation and infiltration of T-cells into the CNS. Despite these findings, several studies have indicated that in early MS lesions, perivascular macrophages can release the neurotrophic factors, leukemia inhibitory factor (LIF) and brain-derived neurotrophic factor (BDNF), suggesting a possible neuroprotective role (Stadelmann et al., 2002; Vanderlocht et al., 2006).

1.5.5 Choroid Plexus Macrophages

A second entry point for immune cells into the CNS is the choroid plexus, the interface between the peripheral circulation and cerebrospinal fluid (CSF). The choroid plexus is responsible for the synthesis of CSF, entry of nutrients into the CSF and the removal of waste products (for review see: (Lun et al., 2015). The choroid plexus also houses the innate immune cells: mast cells, dendritic cells, macrophages and Kolmer`s epiplexus cells. These cells can express adhesion molecules and secrete cytokines. Specifically the Kolmer`s epiplexus cells can act as antigen-presenting cells to lymphocytes (Marques and Sousa, 2015). In healthy individuals it is likely that the choroid plexus directs and controls the immune surveillance of the CNS; however, in pathological conditions, the choroid plexus can become an entry point for large numbers of immune cells into the CNS. In EAE, inflammation in the choroid plexus occurs prior to T-cell

infiltration and lesion formation (Brown and Sawchenko, 2007). Macrophages within the choroid plexus are capable of expressing MHCII and co-stimulatory molecules, possibly leading to the activation of immune cells to CNS antigens. In addition, choroid plexus macrophages can secrete pro-inflammatory cytokines into the CSF, thereby impacting inflammatory processes throughout the CNS (Garabedian et al., 2000). Conversely, there is some suggestion that recruitment of monocytes via the choroid plexus can promote recovery after CNS injury. In spinal cord injury, monocytes that enter the spinal cord at the lesion site had a pro-inflammatory phenotype, whereas those that transverse the choroid plexus were shown to be anti-inflammatory (Shechter et al., 2013). This suggests that the choroid plexus can influence the monocyte phenotype entering the CNS. Therefore, in MS it may be possible to alter the choroid plexus microenvironment to promote the entry of anti-inflammatory cells.

1.5.6 Meningeal Macrophages

In addition to the choroid plexus, the meninges are a critical entry point for immune cells into the CNS during MS (Pikor et al., 2015). The meninges are made up of several layers: the dura mater, arachnoid mater and pia matter. The primary functions of these layers are to protect the CNS from physical damage and allow the flow of CSF around the brain within the subarachnoid space (area between the arachnoid and pia matter). In EAE, preceding the onset of clinical signs, T-cells were observed entering the subarachnoid space via leptomeningeal vessels (Bartholomaus et al., 2009). These T-cells subsequently contacted meningeal macrophages, which caused the induction of an effector T-cell phenotype (Bartholomaus et al., 2009). In addition to macrophages, resident meningeal c-kit^{+} mast cells and dendritic cells are important for the development of EAE disease (Christy et al., 2013; Greter et al., 2005). In the chronic phase of

the disease, meningeal inflammation has been implicated in the cortical damage associated with MS and EAE. In our work, we observe significant amounts of meningeal inflammation surrounding the spinal cord early in EAE (Thorburn et al., 2016). In particular, there is almost always significant meningeal inflammation near the dorsal root entry zone (see Chapter 4, Figure 4.4)

1.5.7 Microglia

In the context of MS, microglia and macrophages are often studied or described as one entity. This occurs because it is challenging to separate these two cell populations with readily available techniques. However, these are very distinct cell types with different origins and roles in MS disease. Microglia colonize the CNS early in development and originate from yolk sac-derived precursors, whereas nearly all other tissue-resident macrophages arise from hematopoietic stem cells (Schulz et al., 2012; Sheng et al., 2015). Furthermore, recent work has indicated that there are nestin^{+} microglia progenitor cells within the CNS and that they are capable of generating new microglial cells (Elmore et al., 2014). In MS, EAE and other CNS diseases it is well established that under inflammatory conditions microglia undergo proliferation and adopt a more compact morphology. These activated microglia have increased phagocytic capacity and release numerous cytokines and chemokines (Stence et al., 2001).

Microglia in the context of MS have been described as having roles in both neuroinflammation and neuroprotection. Activated microglia correlate with the amount of CNS damage and are present in lesions and NAWM (Henderson et al., 2009). In EAE, microglial activation and proliferation precedes the onset of clinical signs and inhibiting microglial function suppresses the disease (Goldmann et al., 2013; Heppner et al., 2005; Ponomarev et al., 2005).

However, microglia are also capable of promoting repair and recovery. The use of transgenic animals to differentiate macrophages and microglia allowed researchers to demonstrate that the infiltrating macrophages drive the primary demyelination, whereas microglia are needed for the clearance of debris and repair (Ajami et al., 2011; Yamasaki et al., 2014). These studies indicated that microglia can influence disease processes, however microglia have also been implicated in the secondary symptoms (pain, anxiety, cognitive impairment) associated with MS and EAE (Olechowski et al., 2010).

1.6 PAIN IN MULTIPLE SCLEROSIS

1.6.1 Prevalence of Pain in MS

There are multiple types of painful syndromes associated with MS. Recently, the overall prevalence of pain in MS has been estimated to be 62.8% based on a systematic review of 17 studies (Foley et al., 2013). However, within this analysis the estimates of pain in the disease ranged from 40% to 86%. The wide variability in these different studies is generally attributed to differences in methods and classification of painful syndromes. This variability aside, pain in MS has been associated with increased disability, disease duration and age. Although MS occurs more frequently in females, the few studies that have examined it suggest that sex does not appear to be a factor in whether pain develops in MS patients (Archibald et al., 1994; Beiske et al., 2009; Foley et al., 2013; Hirsh et al., 2009).

1.6.2 Types of Pain in MS

1.6.2.1 Trigeminal Neuralgia

Trigeminal neuralgia (TN) involves frequent intense pain following the trigeminal innervations in the face, jaw and cheek (Kumar et al., 2013). TN occurs at a much higher rate in people living with MS compared to the general population. The prevalence of TN in MS patients is somewhere between 2-6% (Foley et al., 2013). This prevalence is more than 20 times higher than that found in the general population. In addition, TN in MS patients differs in that it has an earlier age of onset and a greater chance of presenting bilaterally (Foley et al., 2013).

TN in MS patients is postulated to occur as a result of demyelination around the trigeminal nerve after it enters the pons in the brainstem (Cruccu et al., 2009; Solaro et al., 2013). Recently, an MRI study compared MS patients with painful TN and MS patients with trigeminal sensory disruption but who reported no abnormal pain sensations. Using voxel-based 3D analysis, this study found that most MS patients with painful TN had a lesion in the ventrolateral mid pons (Cruccu et al., 2009). In contrast, MS patients without painful TN had lesions affecting other second order brainstem neurons (Cruccu et al., 2009). Electron microscopic analysis of six rhizotomy samples taken from MS patients showed demyelination in the proximal root that extended up to the junction with the distal part of the root. This demyelination was associated with gliosis and immune activation (Love et al., 2001). Biopsies of the nerve in TN patients without MS also show focal demyelination; however, unlike MS patients, few inflammatory processes are observed (Abhinav et al., 2012; Love et al., 2001). Vascular contacts have been observed in MS patients with TN, indicating that 'classical TN' can occur in MS patients and that TN in MS may not be due solely to demyelination.

1.6.2.2 Lhermitte's Sign

Lhermitte's sign presents as a painful 'pins and needles' sensation down the neck and back into the legs. It is usually triggered by movement in the neck. Treatment for Lhermitte's sign is rare as it usually subsides within several weeks (O'Connor et al., 2008). Lhermitte's sign can occur in the absence of MS; however, the prevalence of Lhermitte's sign in MS patients is estimated at approximately 16% (Foley et al., 2013). One study reported that 41% of MS patients reported having Lhermitte's sign at one point in their disease course (Al-Araji and Oger, 2005). The mechanism underlying Lhermitte's sign is similar to that of TN. MRI studies have associated Lhermitte's signs with lesions in the posterior column of the cervical spinal cord (Al-Araji and Oger, 2005).

1.6.2.3 Pain in the Extremities

Neuropathic pain or "dysesthesia" in the extremities of people living with MS is generally described as a burning pain within the legs and feet. It is considered the most common form of neuropathic pain in MS (Foley et al., 2013; O'Connor et al., 2008; Osterberg et al., 2005). This type of pain occurs as the initial symptom in MS in 1-2 % of patients. It has been estimated that 23% of MS patients report having dysesthesia at some point in the course of the disease (O'Connor et al., 2008). It can occur acutely with a relapse, but is usually a chronic form of pain for MS patients (Osterberg et al., 2005). When dysesthesia occurs acutely during a relapse, it is very likely associated with a focal demyelinating lesion within the dorsal column of the spinal cord. While chronic dysesthesia is typically bilateral, acute onset pain is unilateral depending on the location of the lesion. The acute onset of neuropathic pain can be accompanied by numbness and sensory loss. Similar to other symptoms associated with an MS relapse, dysesthesia can

resolve over time. However, it is unclear if acute onset dysesthesia is a predictor or even a risk factor for developing chronic extremity pain as the disease progresses.

Extremity pain in MS includes painful tonic spasms that are a specific type of episodic spasm associated with MS. They usually occur in the limbs and are accompanied by radiating pain. There are multiple triggers for such painful spasms including touch, movement, and heightened emotions and often occur without any defined cause (Maloni, 2000). It has been reported that pain occurs prior to the spasm and it has been suggested that the pain is not caused by the spasm itself (O'Connor et al., 2008). Painful tonic spasms have also been associated with MRI lesions in the brain and spinal regions mediating motor control that results in increased excitability of motor neurons (Truini et al., 2013).

1.7 PAIN IN ANIMAL MODELS OF MS

1.7.1 Pain in PLP-Induced EAE

Aicher and colleagues conducted the first significant examination of neuropathic pain behaviors in an EAE model in 2004 (Aicher et al., 2004). In this study, EAE was induced both actively and through passive transfer using the $PLP_{139-151}$ peptide fragment. PLP-induced EAE generates a relapsing and remitting clinical course, which has increased disability followed by periods of recovery. Prior to the initial peak in motor dysfunction these mice showed hyposensitivity to noxious heat in the tail. However, it was observed that PLP-EAE mice switch and become hypersensitive to the noxious thermal stimuli as the disease progressed into the chronic stages (Aicher et al., 2004). This experiment was done in both male and female mice and both sexes showed a similar degree of thermal hyperalgesia. The authors also noted that the forepaw did not

show an increase in painful responses and that hyperalgesia occurred in the clinically symptomatic areas (Aicher et al., 2004).

1.7.2 Pain in MOG-Induced EAE

Changes in pain sensitivity have also been characterized in the MOG_{35-55} EAE model. In this model, mice develop a significant hypersensitivity to both mechanical and innocuous cold stimuli (Olechowski et al., 2009). Interestingly, these abnormal pain behaviours developed prior to the onset of clinical motor symptoms. These data indicate that processes that occur before demyelination or neuronal injury, such as spinal inflammation and the activation of resident glial cells, are contributing to the early sensory dysfunction in the model (Olechowski et al., 2009). The changes in pain responses in MOG_{35-55} EAE correlate with increases in astrocyte and microglial/macrophage activation, as well as $CD3^+$ T-cell infiltration into the superficial dorsal horn of the spinal cord prior to the onset of motor impairments (Olechowski et al., 2009).

The role of astrocyte activation in the maintenance of neuropathic pain in EAE has recently been shown to be affected by the bradykinin system (Dutra et al., 2013). Specifically, the B1 bradykinin receptor subtype was shown to colocalize with astrocytes in the spinal cord of EAE mice and pharmacological antagonism of B1 receptor function inhibited mechanical and noxious heat hypersensitivity (Dutra et al., 2013). In astrocyte cultures, antagonism of the B1 receptor blocked pro-inflammatory cytokine release and nitric oxide synthase-2 expression, suggesting that the B1 receptor has a direct role on astrocytes and can modulate pain sensitivity in EAE.

In addition to the immune-driven changes in pain sensitivity in the MOG_{35-55} EAE model, pain behaviours have also been associated with dysfunction in the glutamate transporter system

(Olechowski et al., 2010). Using a noxious and persistent pain model, the formalin test, Olechowski and colleagues demonstrated that EAE mice actually exhibit fewer pain behaviors in response to formalin. The decrease in pain behaviors with formalin stimulation was correlated with a decrease in the levels of a spinal glutamate transporter subtype, excitatory amino acid transporter-2 (EAAT-2). Reductions in EAAT-2 levels are postulated to lead to excessive amounts of extracellular glutamate upon intense noxious stimulation (i.e. formalin) that can then activate pre-synaptic, inhibitory, metabotropic glutamate receptors. Olechowski and colleagues demonstrated that acutely increasing glutamate transporter function could normalize the formalin-induced pain behaviours in mice with EAE (Olechowski et al., 2010). In a separate set of experiments, treating MOG_{35-55} EAE mice chronically with the antibiotic ceftriaxone to prevent the reduction in glutamate transporter levels prevented tactile hypersensitivity associated with EAE from developing (Olechowski et al., 2013). These findings highlight an important role of the glutamate transporter and receptor systems in regulating pain sensitivity in EAE.

1.7.4 Pain in Theiler's Murine Encephalomyelitis Virus (TMEV) Infection

MS is restricted to humans and does not naturally occur in other animals. However, a naturally occurring viral infection of the CNS in mice can produce demyelination and MS-like pathologies (Pachner, 2011). The most studied is TMEV and mouse hepatitis virus (MHV). These models have been primarily used to examine the immune response to a persistent neurotropic virus. However, changes in pain sensitivity and cognition have more recently been examined in this model (Lynch et al., 2008).

Similar to the increased pain sensitivity observed in EAE, mice infected with TMEV display hyperalgesia to heat and have a hypersensitivity to mechanical stimulation (Lynch et al.,

2008). Female mice infected with TMEV have a more rapid onset and profound mechanical allodynia compared to male TMEV mice. In either sex, TMEV-infected mice displayed a higher amount of C-fiber innervation within the hind paw. In addition, TMEV decreased mRNA expression of opioid receptors in the spinal cord, which was linked to a reduced effectiveness of opioid treatment in the model (Lynch et al., 2008).

1.7.4 Pain in MBP-induced EAE

Changes in sensory function have also been examined using the MBP Lewis rat model of EAE. Thibault *et al*. (2011) compared rats induced with MBP and rats induced with MBP that was supplemented with cyclosporin A. Supplementing the MBP with cyclosporin A produces a more severe and relapsing disease. Even with the differences in disease progression, both of these EAE models lead to increased mechanical and thermal sensitivity (Thibault et al., 2011). These two models were also characterized using standard treatments for pain. Treatment with acetaminophen had no inhibitory effect on the pain behaviours in the models but first line treatments for neuropathic pain, gabapentin and tramadol, reduced mechanical hyperalgesia. Tramadol also lessened the hypersensitivity to cold stimuli. The serotonin-norepinephrine reuptake inhibitor duloxetine, that has a similar mechanism as tramadol, only prevented cold allodynia (Thibault et al., 2011).

1.8 NEUROTRANSMITTERS AND MS

A possible link between the autoimmune attack in the CNS and behavioural symptoms associated with MS and EAE are changes in the levels of key neurotransmitters. Not only are neurotransmitter changes associated with behavioural symptoms, but they also have immune

modulatory properties. In general, MS and EAE are associated with increased levels of glutamate (GLU) and decreased levels of 5-hydroxytryptamine (serotonin, 5-HT), norepinephrine (NE), dopamine (DA) and gamma-aminobutyric acid (GABA) (Musgrave et al., 2011b). The cerebralspinal fluid (CSF) of people with MS contains lower levels of the metabolites of 5-HT and NE (Stover et al. 1997). Similarly, EAE animals have increased levels of the excitatory amino acid GLU and decreased levels of 5-HT, NE and GABA (Barkhatova et al., 1998, Gottesfeld et al., 1976, White et al., 1983). Treatments that attempt to restore the levels of these neurotransmitters have been shown to reduce the severity of EAE (Bhat et al. 2010, Hofstetter et al. 2005). When interpreting these types of studies, it is important to consider that treatment effects may be in either peripheral nervous system (PNS) or CNS or arise from a direct effect on immune cells.

1.8.1 Glutamate

There are several lines of evidence that suggest that GLU is involved in the pathology of MS and EAE. The primary theory is that an excess of GLU leads to an abundance of excitatory signals that induces excitotoxic death of neurons and glial cells. In brief, excitotoxic mechanisms occur when excessive and prolonged levels of GLU trigger excitatory receptor activity, which leads to increases in intracellular calcium. The breakdown in calcium homeostasis causes a series of events that lead to excitotoxic damage (D'Orsi et al., 2015). MS lesions are associated with increased levels of glutaminase, the enzyme required for the production of GLU. In addition, in genome wide association studies (GWAS), GLU receptor genes have been associated with MS susceptibility (Baranzini et al., 2009). Evidence collected in a 5 year longitudinal MRI spectroscopy study indicated that a higher GLU concentration in NAWM was predictive of

accelerated decreases in grey matter N-acetylaspartate (NAA), a measure of axonal integrity (Azevedo et al., 2014). Beyond excitotoxicity, immune cells can directly respond to GLU. In EAE and MS during active disease, T-cells express high levels of glutamate receptor 3 (Ganor et al., 2003). T-cells taken from a patient with MS also showed enhanced MOG-induced proliferation when cultured in the present of GLU (Sarchielli et al., 2007a).

1.8.2 GABA

GABA is the major inhibitory neurotransmitter in the CNS. Signalling through type A (ion channel) or type B (G-protein coupled) GABA receptors induces neuronal hyperpolarization either via rapid influx of chloride ions or potassium channel opening and decreased adenylyl cyclase activity (Bormann, 2000). In MS and EAE, studies have shown that GABA levels decrease with disease progression (Cawley et al., 2015; Musgrave et al., 2011a). However, there is some suggestion that higher GABA levels are correlated with worse motor performance in people with RRMS (Bhattacharyya et al., 2013). Despite this, most research indicates that reductions in GABA level results in a loss of inhibitory signalling within the CNS. This in turn allows increased firing in demyelinated axons, leading to excessive energy demands and eventually neurodegeneration (Dutta et al., 2006). In addition to its role in the CNS, GABA also has potent immunological effects. Cells of the immune system have the machinery to synthesize, degrade and respond to GABA. Bhat et al. (2010) observed that EAE disease could be improved with the treatment of soluble GABA or GABA agonists (Bhat et al., 2010). They found that T-cells, macrophages and dendritic cells were able to make GABA and secrete GABA via GAD65 and degrade it with GABA transaminase (GABA-T) and GABA transporter type 2 (GAT-2). The beneficial effect of GABA in EAE was primarily mediated through the inhibitory signalling

of GABA-A receptors on the surface of macrophages (Bhat et al., 2010). Although Bhat et al. could not find GABA receptors on T-cells, other studies have reported that GABA has direct effects on T-cells (Bhat et al., 2010). In particular, *in vitro* assays indicate that GABA and GABA-A receptor agonists can inhibit T-cell proliferation and that GABA can induce channel currents in T-cells (Bjurstom et al., 2008; Tian et al., 1999). The actions of GABA inside and outside of the CNS make it an important target to study for the treatment of MS.

1.8.3 Norepinephrine

A major proportion of NE within the CNS arises from the locus coeruleus (LC). Several studies have described reduced levels of NE and LC damage in both EAE and MS samples. Polak et al. found that at 60 days post induction of EAE, the NE levels were reduced by 35% in the frontal cortex and 50% in the spinal cord (Polak et al., 2011). Within the LC, there was a significant increase in the amount of astrocyte staining coupled with a decrease in tyrosine hydroxylase (rate limiting enzyme in NE production) expression (Polak et al., 2011). This work suggests that NE levels are decreased during the chronic phase of MS. Work from our lab shows that at the onset of clinical signs in EAE, NE levels have already started a downward trend (Musgrave et al., 2011a; Musgrave et al., 2011b). However, one study found that NE was increased in CSF samples from people living with MS (Barkhatova et al., 1998). Nevertheless, increasing the amount of NE appears to improve EAE disease progression. Treating EAE with a synthetic NE precursor, L-DOPA, improved clinical outcome and decreased astrocyte reactivity (Simonini et al., 2010). Treatment with venlafaxine (a serotonin-noradrenaline reuptake inhibitor) also has a beneficial effect on EAE induced by adoptive transfer (Vollmar et al., 2008). However, using a selective NE reuptake inhibitor (atomoxetine) after the onset of EAE disease did not provide any

benefit, suggesting that residual NE needs to be present in order for reuptake inhibitor to have any benefit (Simonini et al., 2010).

1.8.4 Serotonin

While 5-HT is best known for its diverse effects within the CNS, it is in fact present throughout the body, with high concentrations in the gut and in platelet cells. 5-HT is synthesized from tryptophan, with tryptophan hydroxylase being the rate limiting enzyme (Walther et al., 2003). There are 2 isoforms of tryptophan hydroxylase, type 1 is localized to enterochromaffin cells of the gut and type 2 is present in central and peripheral neurons (Walther and Bader, 2003). 5-HT is rapidly degraded by monoamine oxidase (MAO) and subsequently an aldehyde dehydrogenase, resulting in the main metabolite 5-hydroxyindole-3-acetic acid (5-HIAA). The breakdown of 5-HT can be prevented through the reuptake and repackaging of 5-HT in vesicles, a process that is dependent on serotonin transporters (SERTs). 5-HT is mainly made by neurons within the raphe nuclei of the brainstem (Soiza-Reilly and Commons, 2014). Serotonergic neurons project from the raphe nuclei throughout the brain and spinal cord. The descending innervation into the spinal cord by 5-HT fibers has been associated with the modulation of nociception and motor function (Perrier and Cotel, 2015).

Like the other neurotransmitters discussed above, 5-HT can have direct effects on immune cells and modulate the inflammatory response. Most components of the immune systems including mast cells, monocytes, neutrophils, natural killer (NK) cells, dendritic cells, T-cells and B-cells have been shown to express 5-HT receptors (Ahern, 2011). Lymphatic tissues are highly innervated with sympathetic neurons that can release serotonin and influence the immune response. In addition, circulating platelets contain stores of 5-HT which are released as a

result of injury and can act as a chemo-attractant (Durk et al., 2013). 5-HT is generally considered to be pro-inflammatory; in particular it is critical for the development of gut inflammation (Ghia et al., 2009). In fact, 5-HT signalling increases T-cell proliferation and may be able to influence T-cell subsets (Inoue et al., 2011; Yin et al., 2006). In the context of MS, blocking either 5-HT1A or 5-HT3 receptor signalling has been shown to improve EAE disease progression (Aminian et al., 2013; Freire-Garabal et al., 2003). Furthermore, EAE disease was attenuated in mice lacking SERT (Hofstetter et al., 2005). Treatment of EAE with the antipsychotic agent risperidone (D2 and 5-HT2A receptor antagonist) decreased the severity of the disease and reduced the amounts of IL-17, IL-2 and IL-4 produced by splenocytes at the peak of disease (O'Sullivan et al., 2014). These studies highlight the modulatory effect serotonin can have on the immune system during EAE.

1.9 MS, EXERCISE, PAIN AND MITOCHONDRIA

1.9.1 MS and Exercise

There has been a recent push to study exercise as a therapy for MS. This is in direct contrast to the previous thinking that physical exercise worsened MS symptoms and therefore should be avoided (Giesser, 2015). This has been proven not to be true; exercise is in fact safe and may provide disease-modifying effects in people living with MS (Pilutti et al., 2014). The most commonly observed benefit, regardless of exercise type (resistance or aerobic), is an improvement in muscle strength (Latimer-Cheung et al., 2013). Although still unclear, several systematic reviews have attempted to decipher whether or not exercise improves the secondary symptoms of MS such as fatigue, cognition and depression. A meta-analysis combining various types of exercise training indicated a small benefit on MS-related fatigue (Pilutti et al., 2013).

Ensari et al. 2014 reviewed the effect of exercise on depression in MS and found an overall positive result with an effect size of 0.36 (Ensari et al., 2014). An initial study showed no benefit of exercise or yoga on cognitive impairment in MS (Oken et al., 2004). However, the exercise regime used in this study was very low intensity (once per week). In contrast, a recent randomized control trial using 2-3 times per week of aerobic exercise at an intensity based on each participant's fitness level indicated an improvement in cognitive function compared to controls (Briken et al., 2014). This highlights the importance of studying various types and intensity of exercise in an attempt to determine the optimal exercise strategy with people with MS. There are only a small number of studies examining the effect of exercise in EAE, and like human studies, they vary in the type and timing of the exercise used. These studies are discussed in greater detail in the introduction and discussion in chapter 4 of this thesis.

1.9.2 Exercise and Pain

Exercise has been found to be an effective treatment strategy to relieve pain in several chronic pain conditions including: chronic neck pain, osteoarthritis, fibromyalgia and chronic low back pain. Pain modulation is a complex interaction between nociceptive input, spinal, and brain processing, and since exercise impacts the whole body, it can influence pain pathways at multiple points. In a sciatic nerve ligation model of neuropathic pain, forced treadmill running reversed the hypersensitivity phenotype through modulation of endogenous opioids (Stagg et al., 2011). In addition, the effects of exercise on pain have been associated with the suppression of substance P and inflammatory cytokines in the dorsal root ganglion (Chen et al., 2014b). Interestingly, exercise can influence neurotransmitter systems within the CNS, thereby possibly improving pain via modulation of local spinal pathways (Martins et al., 2013; Mazzardo-Martins

et al., 2010). Despite the many positive outcomes of exercise and pain, there are several instances where exercise may be less effective. Firstly, in the chronic pain conditions that are characterized by dysfunctional endogenous analgesic pathways, exercise therapy may be less able to harness these pathways to modulate pain symptoms (Van Oosterwijck et al., 2012). Secondly, exercise induces a stress response within the body, resulting in diverse hormonal changes, in particular in the hypothalamic-pituitary-adrenal (HPA) (Droste et al., 2003). There is evidence that the HPA axis is dysfunctional in fibromyalgia and low back pain (Griep et al., 1998). In these conditions, exercise will likely have a minimal impact on the HPA, thereby limiting the effect of exercise on pain. One avenue that has been overlooked, however, is the relationship between exercise, pain and mitochondria. Exercise is known to manipulate mitochondrial content and function, but it is unknown if this is important in the context of exercise and pain.

1.9.3 Mitochondria and Pain

Recently, an association between mitochondrial dysfunction and chronic pain has been pointed out due to studies examining animal models of chemotherapy, HIV and diabetes-associated neuropathies. In animals, several doses of the chemotherapy drug paclitaxel induce significant increases in the amount of atypical mitochondrial found in C-fibers (Barriere et al., 2012). Mitochondrial disruptions have also been seen in oxaliplatin- and bortezomib-induced painful neuropathy (Zheng et al., 2011). Interestingly, mitochondria in the surrounding myelinating and non-myelinating Schwann cells appear to be unaffected. Chemotherapy-induced pain can be improved using reactive oxygen species (ROS) scavengers and via inhibition of complex I and 3 of the electron transport chain (ETC) (Fidanboylu et al., 2011; Griffiths and Flatters, 2015).

Diabetic neuropathy begins with the most distal axons in the feet dying and progresses up the legs (Charnogursky et al., 2014). In animals, diabetic neuropathy is generated by destroying pancreatic beta cells (Biessels et al., 2014). In this model, heat and mechanical hyperalgesia precede complete loss of sensory function (Courteix et al., 1993). Inhibition of the ETC and administration of ROS scavengers can alleviate pain hypersensitivity in these animals (Joseph and Levine, 2006; Kamboj et al., 2010). Interestingly, the diabetic mouse line db/db shows increased numbers of axonal mitochondria in the dorsal root and this increase is associated with mitochondrial biogenesis (Vincent et al., 2010). Mitochondrial dysfunction is also thought to have a role in fibromyalgia. A recent paper found that metformin, a drug commonly used to treat diabetes, caused a 5' adenosine monophosphate-activated protein kinase (AMPK)-dependent improvement in mitochondrial dysfunction in fibroblasts from fibromyalgia patients (Alcocer-Gomez et al., 2015). Interestingly, metformin was also shown to reduce EAE symptoms through modulations of Th17 and Treg cells (Sun et al., 2016). However, this study did not investigate the effect of metformin on mitochondria dysfunction or pain associated with EAE.

1.9.4 Mitochondria in MS

The primary function of mitochondria is to provide the cell with energy in the form of adenosine triphosphate (ATP). However, mitochondria have a central role in many other cellular processes, such as apoptosis, fatty acid oxidation, and calcium homeostasis. In addition, mitochondria are constantly producing ROS, which under normal healthy conditions is buffered by the endogenous cellular antioxidant systems. The CNS requires large amounts of energy, making it particularly susceptible to damage from mitochondrial dysfunction. As a consequence, mitochondrial function is a key factor in the development of several neurodegenerative diseases

such as Alzheimer's, Parkinson's and Huntington's disease. Recent evidence has added MS to this list. In particular, alterations in mitochondrial function have been implicated in the neurodegenerative component of MS. In EAE, mitochondrial injury was shown to be an important step towards axonal degeneration and this was shown to be dependent on the presence of ROS (Nikic et al., 2011).

In MS, glial cells and infiltrating monocytes are likely one of the initial sources of ROS. Oxidative damage is associated with microglia and macrophages expressing NADPH oxidase (Fischer et al., 2013). In autopsy samples oxidized lipids can be found in myelin membranes and oligodendrocytes in areas of active white matter and cortical demyelination (Haider et al., 2011). Astrocytes may also be involved in controlling ROS within the CNS. Depletion of astrocytes in healthy animals caused a disruption of the CNS redox homeostasis, which is sufficient to cause oxidative stress mediated neuronal loss (Schreiner et al., 2015). Regardless of the source of the oxidative stress, a primary consequence of the uncontrolled redox state in the CNS is mitochondrial dysfunction.

Mitochondrial dysfunction has been described in the cortex and white matter of patients with MS (Mahad et al., 2015). Immuno-histochemical analysis of MS lesions has detected changes in mitochondrial respiratory chain proteins in axons, oligodendrocytes and astrocytes (Mahad et al., 2008). Reduced expression of nuclear encoded mitochondrial genes and decreased complex II and complex IV activity has been found in cortical neurons of people with SPMS (Campbell et al., 2011). In addition, a key regulator of mitochondrial function, PGC-1α, has been shown to be reduced in the cortex of MS patients (Witte et al., 2013). As mentioned, ROS are thought to be a key mediator of the mitochondrial dysfunction in MS. In particular, reactive oxygen and nitrogen species are able to induce mitochondrial DNA (mtDNA) damage in MS

(Campbell et al., 2011). These DNA mutations further damage mitochondrial function, which leads to more ROS, thereby creating a cycle of increased ROS and pathology (Witte et al., 2010).

Under normal conditions mutations in mtDNA and damaged mitochondria should be removed in a process called mitophagy. This process requires the expression of PTEN-induced putative kinase 1(PINK1) on the surface of mitochondria, which recruits PARKIN and sets off a cascade that leads to mitochondrial degradation in autophagosomes (Dupuis, 2014). This process also involves proteins important for the fission, fusion and transport of mitochondria. The involvement of these processes in MS is currently understudied. In chapter 5, the involvement of mitochondrial fission, fusion and function in early EAE is investigated and discussed further.

1.10 SUMMARY: OUTLINE AND OBJECTIVES

This thesis is composed of two distinct research projects that are split into 4 experimental chapters. The overall aim of the research described in this thesis was to investigate the underlying mechanisms and assess novel therapeutic strategies to overcome the co-morbid symptoms associated with MS.

The first portion (chapters 2 and 3) of this thesis investigates the contribution of reduced levels of CNS neurotransmitters to EAE disease progression. Specifically, EAE mice were treated with the MAO inhibitor phenelzine (PLZ) to determine whether increasing the levels of NE, 5-HT and GABA could improve EAE associated deficits. The overall hypothesis is that the recovery of neurotransmitter levels in the CNS of EAE mice will reduce the severity of the disease. Moreover, I proposed that PLZ treatment would be effective when given either prior to or after the first signs of EAE disease.

The second portion (chapters 4) of this thesis examines a non-pharmacological approach to improving EAE disease severity. In particular, my interest was in determining whether only one hour per day of exercise could improve EAE-associated pain hypersensitivity. My hypothesis was that daily exercise will improve mechanical hypersensitivity at the onset of clinical signs. I propose that this improvement was due to reduced T-cell infiltration, decreased gliosis, and improved mitochondrial function in the dorsal spinal cord. In a follow-up study (chapter 5), we set out to further investigate mitochondrial dysfunction in the dorsal spinal cord. I hypothesized that within the dorsal spinal cord of EAE mice, mitochondrial function was disrupted by an imbalance in mitochondrial dynamics. To test this hypothesis, tissue samples of dorsal lumbar spinal cord from EAE mice were analyzed for the expression of mitochondrial fission and fusion proteins.

General experimental outline by chapter:

Chapter 2: Investigates whether treating EAE mice daily with PLZ prior to the onset of clinical sign can reduce EAE disease severity.

Chapter 3: Investigates whether PLZ is still effective when given after the onset of clinical signs.

Chapter 4: Investigates whether one hour per day of voluntary wheel running improves EAE-associated pain hypersensitivity.

Chapter 5: Investigates whether mitochondrial dysfunction contributes to the sensory abnormalities associated with EAE.

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Chapter 2

The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE)

2.0 INTRODUCTION

Changes in neurotransmitter concentrations in multiple sclerosis (MS) and the experimental autoimmune encephalomyelitis (EAE) model of MS are recognized to underlie many neurological symptoms associated with the disease, and there is accumulating evidence demonstrating that immune function is directly regulated by the activity of certain neurotransmitters (Bhat et al., 2010; Froh et al., 2002; Hofstetter et al., 2005; Lee et al., 2011; Levite, 2008; Vollmar et al., 2009). We have recently shown that a mouse model of EAE (MOG_{35-55} EAE) is associated with chronic deficits in spinal cord concentrations of norepinephrine (NE), 5-hydroxytryptamine (5-HT/serotonin) and γ-aminobutyric acid (GABA) (Musgrave et al., 2011c). Functionally, these neurotransmitters are both neuro- and immunomodulatory. Furthermore, recent studies have shown that therapeutic agents that increase GABAergic and monoaminergic signalling can lessen the severity of EAE (Bhat et al., 2010; Simonini et al., 2010; Taler et al., 2010; Vansant et al., 2007; Vollmar et al., 2009; Wang et al., 2008b).

Based on its potent ability to increase the CNS concentrations of biogenic amines and GABA (Baker et al., 1984; Baker et al., 1991; Blier et al., 1986; McKenna et al., 1991; McKim et al., 1983; McManus et al., 1992; Paslawski et al., 1995), the antidepressant phenelzine (PLZ) is a promising drug candidate to alleviate the clinical symptoms and neuropathological features of EAE. Clinically, PLZ is effective in the treatment of psychiatric disorders like atypical depression, social anxiety disorder and panic disorder (Baker et al., 2007). PLZ inhibits monoamine oxidase (MAO), the enzyme responsible for the degradation of biogenic amines, as well as GABA transaminase (GABA-T), the enzyme responsible for degradation of GABA (Baker et al., 2007). PLZ is also metabolized by MAO, and one of the resultant metabolites,

phenethylidenehydrazine (PEH), inhibits GABA-T and is thought to make a major contribution to the marked elevations of brain GABA concentrations produced by PLZ (MacKenzie et al., 2010a; MacKenzie et al., 2010b; Paslawski et al., 2001).

It is now recognised that MS is also associated with non-motor symptoms such as pain, fatigue, cognitive impairment and depression (Compston and Coles, 2008; O'Connor et al., 2008; Peruga et al., 2011; Thibault et al., 2011). Like MS, EAE models are also associated with these 'non-motor' behavioural changes (Olechowski et al., 2009; Peruga et al., 2011; Pollak et al., 2002; Pollak et al., 2000). Evaluating behavioural changes in addition to the more common measures of motor impairment may be critical if EAE is to be fully considered in the context of the human condition it models. While the efficacy of PLZ in tests of panic, anxiety and depression in animals is well established (Bourin et al., 2002; Griebel et al., 1998; Maki et al., 2000; Paslawski et al., 1996; Zhao et al., 2009), it has not been examined in the context of a disease such as EAE. Physiologically, chronic treatment with PLZ may confer additional neurological benefits through modifications of excitatory neurotransmitter release and reuptake as well as affecting the HPA axis (Balu et al., 2008; Kier et al., 2005; Michael-Titus et al., 2000; Sands et al., 2004; Song et al., 2010; Tanay et al., 2001b). Long-term inhibition of MAO also suppresses the production of pro-inflammatory cytokines, which may reduce the intensity of motor and affective changes in EAE (Bielecka et al., 2010; Lin et al., 2000). Therefore we set out to evaluate the effects of chronic PLZ treatment on affective and motor aspects of EAE disease progression. We show here that daily treatment with PLZ can improve functional outcomes and attenuate disease severity in mice with EAE.

2.1 METHODS

2.1.1 EAE Induction

All animal procedures were performed according to protocols approved by the University of Alberta Health Sciences Animal Care and Use Committee. EAE was induced in female C57/BL mice (Charles River) with MOG_{35-55} . EAE was induced by subcutaneous injection of 50 μg of myelin oligodendrocytes glycoprotein (MOG_{35-55}) emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1mg/mL. Control mice received subcutaneous injections of CFA alone. An intraperitoneal injection of 300 ng pertussis toxin (Sigma-Aldrich, Oakville, ON) was administered on the day of induction and 48 hours later. Mice were monitored daily for clinical signs of EAE and were graded on using a 5 point scale.

2.1.2 EAE Assessment.

Mice were monitored daily for clinical signs of EAE and were graded according to the following scale: Grade 0, normal mouse; Grade 1,flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both (Kalyvas and David, 2004).

2.1.3 Drug treatments.

For the evaluation of PLZ's acute effects on neurotransmitter concentrations in the CNS, PLZ was administered to mice with EAE at the "Peak" stage of disease progression (clinical score of grade 3 or higher) or to controls (CFA) via a single, intraperitoneal (IP) injection at a dose of 30 mg/kg in bacteriostatic water according to PLZ's free base weight. Three hours after injection, a time at which target concentration changes in GABA have neared their peak elevation

(MacKenzie et al., 2008; Tanay et al., 2001a; Wood et al., 2006a), mice were euthanized and regions of the CNS were extracted and frozen. For the experiment assessing the effects of daily treatment with PLZ in EAE, mice were given daily IP injections of vehicle or PLZ at a dose of 15 mg/kg beginning 7 days after disease induction. Treatment lasted for 28 days. This dose is approximately 10 times higher, on an mg/kg basis, than the dose used in humans but given the much higher metabolic rate of rodents compared to humans, higher doses are often used in rodent studies. Detailed pharmacokinetics of phenelzine in humans are not available for comparison of human and rodent plasma levels, but a dose range of 10-30 mg/kg i.p. for rodents has been used frequently in the literature (Baker et al., 1984; Balu et al., 2008; Paslawski et al., 1996; Zhao et al., 2009). At this dose, lethargy and anorexia, which are sometimes observed in PLZ-treated rodents, are minimized and still maintain full behavioural and neurochemical effects (Griebel et al., 1998; Parent et al., 2000; Paslawski et al., 1996; Zhao et al., 2009). Injections were given in the afternoon after behavioural assays were completed. CNS tissue was taken for immunocytochemistry or HPLC analysis the morning after the last injection.

2.1.3.1 PLZ acute effects

PLZ was administered to mice with EAE at the "Peak" stage (clinical score of grade 3 or higher) or to controls (CFA) via a single, intraperitoneal (IP) injection at a dose of 30 mg/kg. Three hours after injection mice were euthanized and regions of the CNS were extracted and frozen.

2.1.3.2 PLZ chronic effects

For the experiment assessing the effects of daily treatment with PLZ in EAE, mice were given daily IP injections of vehicle or PLZ at a dose of 15 mg/kg beginning 7 days after disease induction. Treatment lasted for 28 days.

2.1.4 Behavioural Assays.

Rotorod-Locomotor abilities were assessed using a protocol previously employed by our lab (Olechowski et al., 2009). Mice were given three days of training on the rotorod before disease induction. Trials were conducted, starting the second day after EAE induction, every second day until day 22, after which rotorod abilities were tested on only days 29 and 36. Naive, CFA and EAE mice were placed for 3 minutes on the rotating beam of a rotorod (MED Associates Inc., ENV-576M) that was rotating at a fixed rate of 16 revolutions per minute. Each mouse was given three trials, after which the average time a mouse remained on the rotating beam was calculated. ***Open field assay***-Open field analysis was conducted in the morning on the same days as the rotorod using a clear plastic container (width: 29 cm, length: 44 cm, height: 17 cm) placed on a grid that divided the box into four quadrants of equal size. Mice were observed in the open field for 4 minutes, during which the behaviours being evaluated were recorded. The open field was cleaned and wiped with ethanol after each cage of mice completed their trials. A standard electronic timer was used to keep time. No pre-induction habituation was conducted in the open field. In another study, 3 days of habituation in the open field produced similar patterns of behavioural changes after the EAE induction procedure (unpublished observations).

In each trial in the open field, the number of times a mouse crossed into an adjacent quadrant (Crossing) was recorded and a score corresponding to the amount of time the mouse spent in a specific sedentary posture (Activity/Attention Score) was given. The number of crossings, noted each time the mouse moved into an adjacent quadrant with all four limbs, was considered as a measure of exploratory behaviour and as an objective indicator of the mouse voluntarily attending to cues of interest within its environment. The Activity/Attention Score is a categorical score given each minute to a mouse based on the duration it spends in a specific

sedentary posture. This posture was defined as having both forepaws on the ground and with the head being relatively still with a steady gaze directed below the horizontal (i.e. a head position with the nose pointing towards the floor of the observation box). This score was developed because, while all mice spend considerable amounts of time in one quadrant of the open field, EAE mice were often observed to adopt this posture. These postures are never observed in naive animals and only rarely in control mice immunized with CFA alone. This posture is reminiscent of sickness behaviour in rodents, as described by Dantzer et al. (2008), and is suggestive of a disinterest in cues from the physical environment and from itself, as indicated by grooming. The total duration spent in this posture was measured using a standard stop watch and scores were given each minute according to the following criteria: 0, mouse is still and with a floor-directed gaze for 45 or more seconds in one minute; 1, mouse is still and with a floor-directed gaze for 30 to 45 seconds in one minute; 2, mouse is still and with a floor-directed gaze for 15 to 30 seconds in one minute; 3, mouse is still and with a floor-directed gaze for 0 to 15 seconds in one minute. These scores were totalled for each mouse at the end of the 4-minute open field trial. Timing was stopped if the mouse interrupted this posture for any reason, including extensions of the head/neck forward or upward (usually to sniff at the air); large, lateral investigatory head movements, or initiation of grooming. Small head movements and minute shifts in weight were tolerated, however, so long as the direction of gaze did not change. In addition to CFA controls, naive mice were also examined in this experiment's behavioural assay.

2.1.5 Histology and Immunocytochemistry.

Histological analysis was carried out on spinal cord tissue from CFA controls and mice with EAE after chronic treatment with PLZ. Mice were anesthetised and sacrificed by transcardiac

perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The lumbar enlargement of the spinal cord was removed, post-fixed for 3–4 h and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm).

To assess myelin loss, sections were stained with Eriochrome Cyanine (EC) following a standard protocol. To assess inflammation and reactive gliosis, a rat anti-CD3 and rat anti-Mac1 were used (Cd11b) (1:200, Serotec, Oxford, UK). Changes to 5-HT in the spinal cord were observed using a rabbit anti-5-HT (1:5000, Sigma) antibody. In order to evaluate the amount of axonal loss, spinal cord tissue sections were incubated with a cocktail of SMI-32 (1:500 , Covance, Cedarlane Labs, Burlington, On) and SMI-312 (1:500 , Covance, Cedarlane Labs, Burlington, On) mouse monoclonal antibodies. These were visualized using goat anti-rat Alexa Fluor®594 or 488 (1:200, Molecular Probes, Eugene, OR)..

5-HT primary antibody was visualized using a goat anti-rabbit Alexa Fluor®488 secondary antibody (1:200, Molecular Probes, Eugene, OR). In order to evaluate the amount of axonal loss, spinal cord tissue sections were incubated with a cocktail of SMI-32 (1:500 , Covance, Cedarlane Labs, Burlington, On) and SMI-312 (1:500 , Covance, Cedarlane Labs, Burlington, On) mouse monoclonal antibodies. SMI-312 recognizes the phosphorylated form of 200-kd neurofilament subunit (NF200) occurring in healthy axons and SMI-32 recognizes dephosphorylated NF200 occurring when axons become damaged. Areas lacking both the dephosphorylated and phosphorylated NF200 subunit were used as markers of axonal loss. The primary antibody was visualized using a goat anti-mouse Alexa Fluor®594 secondary antibody (1:200, Molecular Probes, Eugene, OR).

2.1.6 Quantification of histology and immunohistocytochemistry.

Images were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All image analysis was carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. The number of CD3+ positive cells within the parenchyma of the ventral spinal cord was counted in spinal segments from the lumbar spinal cord (L4-L5 segments). An average number of CD3+ T-cells was calculated per section (3 sections per slide, two slides per animal, n=5 animals per group). Optical density measurements of Mac1(Cd11b) immunoreactivity were examined in the same region of ventral spinal cord (3 sections per slide, two slides per animal, n=5 animals per group). The level of background staining was determined for each section and subtracted for all optical density measurements. The innervation density of 5-HT was assessed in cross-sections of the lumbar spinal cord from L4 and L5 segments. A standardised area of ventral horn grey matter was assessed in tissue sections from CFA, EAE, CFA+PLZ and EAE+PLZ mice (3 sections per slide, two slides per animal, analyzing both ventral horns per section, n=5 animals per group). Staining density was measured using NIH ImageJ software using a manual threshold function to quantify the optical density of staining within the standardised area of ventral horn from each section. Myelin loss was defined as the absence of EC staining within a spinal cord section. An area of axonal loss was determined by the absence of both SIM-32 and SMI-312 staining. Myelin and axonal loss were quantified using NIH ImageJ software and determined by measuring the total area devoid of staining. The sum of the unstained areas within a section was calculated and expressed as a percent of the total white matter area in that section.

2.1.7 High Performance Liquid Chromatography (HPLC).

All water used was distilled and purified by reverse osmosis using a Milli-Q filtration system from Millipore. Methanol (MeOH), tetrahydrofuran (THF), and acetonitrile were HPLC grade and were all obtained from Fisher Scientific. Solvents were filtered using Millipore nylon membranes (0.2 μm pore size). *o*-Phthaldialdehyde (OPA) and ascorbic acid were obtained from Sigma Aldrich, N-isobutyryl-L-cysteine (IBC) from Novachem and sodium borate from Fisher Scientific.

Standards and tissue samples were analyzed for amino acids using a minor modification of the procedure of Grant et al. (2006). Tissues were homogenized in 5 volumes of ice-cold MeOH, left on ice for 10 minutes and then centrifuged at 10,000xg for 4 minutes. The resultant supernatants were then diluted in water to make a 30- or a 60-fold dilution. Portions of these diluted supernatants were then reacted with OPA and IBC and the resultant derivatives used for analysis, with the fluorescence detector set at an excitation wavelength of 344 nm and an emission wavelength of 433 nm. Calibration curves prepared from authentic samples of amino acids were generated for each individual run of samples.

The procedure of Parent et al. (2001) was used for the separation and analysis of biogenic amines and their metabolites. To aliquots of tissue in 5 volumes of water, 1/10 the volume of ice-cold 1N $HClO_4$ containing ascorbic acid (500μM) and EDTA (100 mg %) was added. These homogenates were vortexed, centrifuged at 10,000xg for 4 minutes and the supernatant was removed for analysis by HPLC. The applied potential for electrochemical detection was 0.65 V. Calibration curves were constructed for each HPLC run.

2.1.8 Statistical Analysis.

Statistical analyses were conducted using SigmaPlot software version 11.0. HPLC results were analysed using Student's t-tests. Two-way repeated measures ANOVA was used to compare clinical scores, rotorod, and open field scores between treatment groups and Tukey's post-hoc test was utilized to reveal specific points of difference between groups. For the analysis of activity scores, the scores of CFA mice treated with PLZ or vehicle were pooled because deviations from scores of 12 were rare in both groups. In all instances, results were considered as statistically significant if $P<0.05$.

2.2 RESULTS

2.2.1 Changes in GABA, 5-HT and NE levels in EAE mice after acute treatment with PLZ

It is well established that acute treatment with PLZ can significantly increase the levels of 5-HT, NE and GABA in the nervous system (Baker et al., 2007; Parent et al., 2000). In mice with EAE, there are significant changes in the levels of these neurotransmitters, primarily in the spinal cord, but also in more rostral structures in the brain and brainstem (Krenger et al., 1986; Krenger et al., 1989; Musgrave et al., 2011c). To determine whether PLZ could normalize or enhance 5-HT, NE and GABA levels in the CNS of mice with EAE, we treated animals at the peak stage of the disease with a single, acute dose of PLZ, shown in our previous work to increase the levels of all three neurotransmitters in control mice. Using HPLC, we measured the levels of 5-HT, NE and GABA in the spinal cord, brain, brainstem, cerebellum and cerebrum (rest of brain) after this treatment (see Table 2.1 A,B for concentrations). In CFA control mice receiving PLZ, the effect of PLZ treatment appears to vary between the CNS regions examined. The percentage increase in concentration for 5-HT varied between 54% in the cerebrum to nearly 300% in the brainstem

(Fig. 2.1C,D). The effects on NE levels in CFA mice were milder, ranging from an approximate 50% increase in the brainstem and cerebellum to a 117% increase in the cerebrum (Fig. 2.1F,G,H). However, the effects of PLZ on GABA concentrations were relatively consistent, ranging from an increase of 110% in the cerebrum to a 132% rise in the brainstem of CFA mice (Fig. 2.1J,L).

PLZ had a more dramatic effect on the levels of 5-HT, NE and GABA in the CNS of mice with EAE. Concentration increases in 5-HT after PLZ administration were significantly more robust in the spinal cords and cerebra of EAE mice compared to CFA controls. 5-HT levels rose nearly 1000% in the spinal cord of EAE mice receiving acute PLZ relative to untreated EAE mice (Fig. 2.1A). There was a also a greater increase in NE and GABA levels in the spinal cord of EAE mice where levels rose by approximately 300% compared to untreated EAE mice (Fig. 2.1E,I). Overall, the relative effectiveness of PLZ to increase 5-HT, NE and GABA concentrations was significantly greater in the EAE spinal cord than in non-diseased, CFA controls (*5-HT Spinal Cord*-CFA+PLZ: 99.27 ± 3.80 % change from CFA vs. EAE+PLZ: 972.63 ± 153.10 % change from EAE, P≤0.001, t-test; *NE Spinal Cord*-CFA+PLZ:104.35 ± 12.60 % change from CFA vs. EAE+PLZ : 309.21 ± 44.56 % change from EAE, P=0.002, t-test; *GABA Spinal Cord*-CFA+PLZ: 122.79 ± 8.53 % change from CFA vs. EAE+PLZ: 282.10 ± 23.19 % change from EAE, P≤0.001, t-test) (Fig. 2.1A,E,I). In the cerebrum, PLZ was more effective at elevating 5-HT and GABA in EAE mice than non-diseased controls (*5-HT Cerebrum*-CFA+PLZ: 54.25 ± 1.82 % change from CFA vs. EAE+PLZ: 88.90 ± 10.33 % change from EAE, P=0.011, t-test; *GABA Cerebrum*-CFA+PLZ: 117.64 ± 13.48 % change from CFA vs. EAE+PLZ: 220.37 ± 9.38 % change from EAE, P<0.001, t-test) (Fig. 2.1D,L). However, in the cerebrum, NE levels were increased more in non-diseased CFA controls than EAE mice (*NE*

Cerebrum-CFA+PLZ: 116.87 ± 2.75 % change from CFA vs. EAE+PLZ: 47.86 ± 11.57 % change from EAE, P<0.001, t-test) (Fig. 1H).

2.2.2 PLZ improves functional outcomes in EAE

Some antidepressants have been shown to alleviate many of the clinical and neuropathological features of EAE (Bhat et al., 2010; Simonini et al., 2010; Taler et al., 2010; Vollmar et al., 2009) but to our knowledge PLZ has not been investigated in this regard. Therefore, we next assessed how chronic daily treatment with PLZ affected the clinical severity and behavioural signs indicative of anxiety, sickness and/or depression in EAE mice. PLZ treatment (15mg/kg/day) began on the 7th day after EAE induction. PLZ had a significant effect on the motor impairments that are characteristic of EAE disease progression (Fig. 2.2A,B). While PLZ-treated mice reached the same peak clinical score, the onset of clinical signs was significantly delayed and the disease burden was reduced in the chronic phase in EAE mice receiving daily PLZ treatment (P=0.005, two-way RM-ANOVA)(Fig. 2.2A). Gross locomotor ability, assessed by performance on a non-accelerating rotorod, was also better maintained in EAE mice receiving daily PLZ treatment (P=0.001, two-way RM-ANOVA) (Fig. 2B).

Open field behaviours were also significantly improved in EAE mice treated with PLZ. In EAE animals, the amount of line crossings in the open field decreased dramatically in the initial days after disease induction and well before the appearance of clinical signs indicative of disease onset (Fig. 2.2C). In EAE mice receiving daily vehicle injections, these exploratory behaviours continued to decline during the course of the experiment. All mice were capable of general locomotion, as mice were observed to move to the periphery upon being placed in the center of the open field. PLZ treatment had an immediate and pronounced effect on open-field

line crossing in EAE mice when compared to EAE animals receiving vehicle ($P<0.001$ two-way RM-ANOVA) (Fig. 2.2C). However, PLZ treatment did not produce a lasting effect, as there was a sudden decline in the amount of open-field line crossings after approximately 10 days of treatment.

In addition to monitoring exploratory behaviours in the open field, discrete postural changes that served as adjunct measures for how the mice attended to and interacted with their environment were also monitored in EAE and EAE-PLZ treated mice (see **Materials and Methods**). Non-diseased mice rarely had scores below 10-11 using this rating system, consistently paying attention to their surrounding environment (Fig. 2.2D). In vehicle-treated EAE mice, the first deviations from a healthy score of 12 develop around the time when the first clinical signs of the disease become apparent ('disease onset'-days 8 -12). The mice become more sedentary compared to non-diseased control mice and often exhibit a posture where they are still, with a floor-directed gaze for a large part of the time they are in the open-field. The activity/attention score continues to drop as the disease progresses. Vehicle treated EAE mice had significantly reduced activity/attention scores beginning on day 16 after EAE induction that persisted for the duration of the experiment ($P<0.001$, Kruskal-Wallis one-way ANOVA, Dunnett's Post hoc test) (Fig. 2.2D). In contrast, the activity/attention scores of EAE mice treated with PLZ were maintained at CFA levels until day 18. Activity/attention scores were significantly greater in PLZ treated EAE mice compared to vehicle treated mice from day 16 until day 22 ($P<0.05$, Mann-Whitney Rank Sum test). However, by the end of the experiment (day 36), both groups had equally reduced activity attention scores (Fig. 2.2D).

2.2.3 Daily PLZ does not affect inflammation and reactive gliosis in EAE

To assess the underlying cellular mechanisms for these improvements in functional outcomes in PLZ treated mice, we began by assessing how treatment affected the influx of T-cells into the CNS parenchyma, a hallmark feature of EAE pathology. In spinal cord sections from vehicle- and PLZ-treated EAE mice, we found no significant difference in the mean number of CD3+ T cells that had infiltrated into the spinal cord (Fig. 2.3A-C). Similarly, when we examined the degree of microglial/macrophage reactivity in these regions, PLZ had not significantly attenuated these responses in diseased mice (Fig. 2.3D-F).

2.2.4 Daily PLZ does not affect demyelination and axonal injury in EAE

Although daily treatment with PLZ did not affect the global immune response in EAE mice, we next wanted to assess whether it had any influence on the downstream outcomes of these inflammatory processes by examining the degree of demyelination and axonal injury. Eriochrome C staining for myelin revealed significant regions of demyelination throughout the spinal cord of vehicle- and PLZ-treated mice with EAE. Demyelination was most prominent in the dorsal and ventral columns (Fig. 2.4A-D). Although demyelinated areas in these regions tended to be slightly smaller in PLZ-treated mice, there was no statistical difference in the overall amount of demyelination between the two groups (Fig. 2.4E).

We next used a cocktail of antibodies that recognise both the phosphorylated and non-phosphorylated forms of neurofilaments to detect healthy and damaged axons in the spinal cord of vehicle- and PLZ-treated mice. Areas completely devoid of staining would therefore represent regions where significant axon loss has occurred. These ‘black holes’ (regions devoid of any axons) were prominent in both groups of mice (Fig. 2.5A-D). In general, the magnitude of these

types of lesions were smaller in PLZ-treated spinal cords, but these effects were not significant, suggesting that PLZ did not have a general neuroprotective effect in these mice (Fig. 2.5E).

2.2.5 Daily PLZ restores 5-HT innervation to the ventral horn of the spinal cord in EAE mice

It is well established that EAE results in a loss of descending 5-HT innervation to the spinal cord (Krenger et al., 1986; Musgrave et al., 2011c; White et al., 1990; White et al., 1985). Reduced 5-HT inputs to motor neuron pools in the spinal ventral horn is a major factor leading to the functional losses in the disease. Given that a single, acute injection of PLZ could elevate 5-HT levels so dramatically in the spinal cord (Fig. 2.1), we next examined 5-HT levels in cross sections of the ventral horn of the spinal cord after chronic daily treatment in mice with EAE. In vehicle-treated EAE mice, the amount of 5-HT immunoreactive fibers in the ventral horn was significantly reduced compared to CFA controls ($P<0.001$ one way ANOVA) (Fig. 2.6A,B,E). However, after chronic daily treatment with PLZ, we found that 5-HT levels were normalised back to control levels in the ventral horn of EAE mice (Fig. 2.6A,C,E).

2.2.6 Chronic PLZ administration elevates biogenic amine levels throughout the CNS of EAE mice but no longer enhances GABA concentrations.

To confirm our histological findings we conducted an additional experiment using HPLC to assess the levels of 5-HT, NE and GABA at different levels of the CNS after chronic daily treatment for 28 days with PLZ (see Table 2.2 for concentrations). As expected, in the spinal cord we found a significant increase in 5-HT and NE levels. 5-HT levels were nearly 8-fold higher in PLZ-treated EAE mice after chronic daily treatment and NE levels were nearly doubled

(P<0.05, t-test) (Fig. 2.7A,E). Chronic treatment with PLZ lead to increased 5-HT and NE levels throughout the CNS with significant increases in the brainstem, cerebellum and cerebrum when compared to vehicle-treated EAE mice (P<0.05, t- test) (Fig. 2.7B-D; F-H). Interestingly, unlike an acute treatment with PLZ, GABA levels were no longer significantly different between vehicle or chronically treated PLZ-EAE mice (Fig. 2.7I-L). This inability of chronic PLZ treatment to maintain a sustained elevation in GABA levels may be an explanation for PLZ's lack of efficacy on disease outcomes in the later stages of the disease (see ***Discussion*** below).

2.3 DISCUSSION

Our results demonstrate the ability of the non-selective MAO inhibitor, PLZ, to attenuate EAE disease severity. These findings add to the growing body of research that demonstrates the efficacy of antidepressants to modify the disease course of EAE and strongly suggest that MAO inhibitors such as PLZ possess therapeutic potential in diseases like MS. While no differences were apparent in the severity of peak clinical scores from vehicle- or PLZ-treated mice with EAE, mice treated with PLZ had a substantially delayed disease onset and reduced overall disability at the end of experiment. The benefits of PLZ treatment in EAE also extended beyond traditional measures of locomotor output in EAE mice. PLZ treatment had beneficial effects on the behavioural signs of anxiety, sickness and/or depression exhibited in the open field by mice with EAE. Additionally, the response to PLZ by EAE mice in their activity/attention scores suggests the potential utility for this easy and novel measure of-sickness and depressive behaviour.

PLZ exerts strong effects on CNS concentrations of 5-HT, NE and GABA (Parent et al., 2000). The concentrations of these neurotransmitters decrease during EAE, with the changes to

spinal cord concentrations of 5-HT and NE being especially dramatic because of substantial damage to descending tracts from the brainstem (White et al., 1990). It is therefore difficult to point to an individual mechanism or pathway responsible for the improvements in functional outcomes seen in EAE mice treated with PLZ as treatment effects may also occur in the peripheral tissues. However, previous studies evaluating the effects of 5-HT, NE and GABAergic neurotransmitter systems on EAE and inflammation may offer clues. Bhat et al. (2010) recently demonstrated that GABAergic agents powerfully reduce EAE severity by inhibiting pro-inflammatory mechanisms, effects that were mediated by GABA receptors present on antigen-presenting cells (Bhat et al., 2010). The delayed disease onset in our experiment may reflect a similar GABAergic suppression of inflammatory mechanisms. Using mice that lack 5-HT transporters, Hofstetter et. al. (2005) showed that increased 5-HT activity reduces EAE disease severity, is associated with fewer inflammatory infiltrates and decreases production of IFNγ (Hofstetter et al., 2005). It has also been demonstrated that increasing NE concentrations after EAE onset, using a combined treatment with the synthetic NE precursor L-threo-3,4-dihydroxyphenylserine and the NE reuptake inhibitor atomoxetine, also improves EAE clinical scores. These treatments did not modify T cell production of interferon-γ (IFNγ) or interleukin 17 (IL-17), but their influence on antigen-presenting cells was not evaluated (Simonini et al., 2010). Finally, treating EAE mice with venlafaxine, which inhibits the synaptic re-uptake of both 5-HT and NE, also suppressed successfully the progression of EAE clinical scores (Vollmar et al., 2009). Therefore, an additive anti-inflammatory response to elevations in the levels of these neurotransmitters may occur in response to PLZ treatment. This would result in less neuronal or axonal degeneration and promote better functional outcomes. However, while there is a trend towards decreased inflammation, axon and myelin damage in our results, we found no significant

differences in these histopathological markers of inflammation, demyelination or axonal injury between vehicle- and PLZ-treated mice with EAE. It remains possible however that PLZ can influence the phenotype of CD4+ T Cell subtypes, thus influencing inflammatory processes in a more subtle manner.

An alternative mechanism for the improved functional recovery of PLZ-treated mice with EAE may be through the elevated concentrations of monoamines such as 5-HT in the ventral horn of the spinal cord. By normalising 5-HT levels to ventral horn motor neuron pools, PLZ could mask the normal accumulation of disability by maintaining neuronal excitability in the face of declining spinal concentrations of biogenic amines as a result of the disease. One caveat to these observations is that our results may not necessarily directly extend to male animals, as only female mice were included in this study and the effects of EAE and antidepressant treatment can differ between genders (Keers and Aitchison, 2010; Voskuhl and Palaszynski, 2001). However, these results are interesting in light of the fact that MS affects females disproportionately to males.

We found that daily PLZ treatment could significantly delay the onset of clinical signs in EAE but did not suppress the disease completely as the peak severity of clinical deficits was not different between PLZ- and vehicle-treated mice. These findings might specifically implicate the GABAergic system's influence during EAE. Our results may reflect a dysinhibition of pathological immune mechanisms. Earlier studies using a GABA receptor agonist or an irreversible GABA-T inhibitor in EAE have demonstrated that the suppression of clinical scores by GABAergic agents may lose its absolute potency with time (Bhat et al., 2010). One possibility is that the enhanced GABAergic activity in response to treatments such as PLZ causes a desensitization of GABA receptors and/or an induced change in their cell surface expression

(Barnes, 1996). Another factor may be involved in the case of PLZ since, as mentioned previously in this paper, it is a substrate for as well as an inhibitor of MAO (Clineschmidt and Horita, 1969b, d; Popov and Matthies, 1969b), with PEH, a metabolite produced by the action of MAO on PLZ, making a major contribution to the GABA-elevating actions of PLZ (MacKenzie et al., 2010a; MacKenzie et al., 2010b; Paslawski et al., 2001). Successive daily treatments with PLZ causes a progressively greater inhibition of MAO, which reduces the conversion of PLZ to PEH and causes progressively weaker inhibition of GABA degradation and an eventual return of brain GABA levels to pre-drug treatment levels. Eventually, this reduced capacity of PLZ to elevate GABA levels would become insufficient at suppressing inflammation and disease progression. Our findings using HPLC on tissue samples after chronic PLZ treatment confirm this hypothesis. We have shown that after chronic daily treatment with PLZ, GABA levels in PLZ-treated EAE mice are no longer potentiated but instead are the same as vehicle-treated EAE levels by the end of the experiment. These findings suggest a likely mechanism for the decline in open-field activity/attention scores and the progression of clinical deficits in PLZ-treated mice observed here.

In addition to assessing classical motor disturbances, our assessment of behaviour in an open field suggests that the affective state of mice is disturbed during the progression of EAE. Previous studies using various methods to analyse the behaviour of animals with EAE have shown changes indicative of depression and anxiety (Peruga et al., 2011; Pollak et al., 2002; Pollak et al., 2003a; Pollak et al., 2003b). For example, Pollak et al. demonstrated the presence of depressive-like symptoms in EAE mice such as decreased social interest and reduced interest in a pleasurable stimulus, a sign of anhedonia (Pollak et al., 2002; Pollak et al., 2000). Additionally, using a mild EAE model characterized by little motor impairment, Peruga et al.

(2011) recently demonstrated that EAE mice show increases in signs of anxiety and depressive behaviours. Using the measures described here, we can observe large behavioural deviations from control levels throughout the time course of our EAE model, and these changes respond strongly to treatment with PLZ. Future studies treating EAE with PLZ that employ more specific assays for detecting anxiety and depression may help better distinguish the nature of the changes we observe.

Depression-like behaviours in EAE are associated with the increased activity of pro-inflammatory cytokines in the CNS (Peruga et al., 2011; Pollak et al., 2003a). The activities of TNFα and IL-1β on their corresponding receptors are particularly implicated, as they can induce depressive behaviours in animals and people as part of their initiation of sickness behaviour (Dantzer et al., 2008). Consistent with idea that endogenous production of proinflammatory cytokines can induce sickness and depressive-like signs in animals, earlier studies of EAE-associated behavioural syndrome demonstrated that these behavioural changes in EAE were best explained by the temporal correlation between behaviour, immune cell infiltration into the brain and the production of IL-1β, TNFα and prostaglandin E_2 (Pollak et al., 2002; Pollak et al., 2003a). Similar behavioural changes have also been observed in the mouse model of another autoimmune disorder, systemic lupus erythematosus (SLE), and include anhedonia, reduced locomotion and exploration in an open field, decreased novel object exploration and cognitive dysfunction. Depressive-like behaviours are evident in these mice early, when signs of immune system activation become evident in the serum (Sakic et al., 1997; Sakic et al., 1994; Szechtman et al., 1997). Notably, the behaviour of EAE mice may also be influenced by the chronic stress associated with having EAE, as chronic stress can cause anxiety and depressive-like behaviour (Heesen et al., 2007).

Our results using these open-field assays are also important because they show that the affective state during EAE is sensitive to antidepressant treatment with PLZ. In EAE mice, PLZ treatments produced immediate, but transient, increases in the amount of exploratory behaviours along with maintenance of activity/attention scores. This transience could derive from the temporary suppression of EAE mechanisms by PLZ. This effect may be mediated by the early elevations of GABA levels by PLZ that are not sustained with chronic treatments (see above). In addition, selective inhibition of MAO-A by moclobemide has been shown to reduce cytokine expression *in vitro*, suggesting that beneficial effects of MAO inhibition on both standard EAE disease mechanisms that lead to locomotor deficits and sickness behaviour in the model are possible (Bielecka et al., 2010; Lin et al., 2000). While there appears to be lasting, mild improvements in exploratory behaviour, the failure of PLZ to elicit statistically significant improvements in the late exploratory behaviours of EAE mice suggests that the benefits of increased monoamine concentrations on this measure are relatively mild in the later time points of EAE. The beneficial effects of elevated monoamine concentrations on sickness and/or depressive-like behaviours, however, may be interpreted from the activity/attention scores of EAE mice treated with PLZ because reductions in these scores occurred at a much slower rate than the accumulation of motor disability after disease onset.

Our results demonstrating improved open field behaviours after PLZ treatment in EAE mice are consistent with the earlier studies in which the antidepressant imipramine or anti-inflammatory treatments produced improvements in the depression-like behaviours of EAE mice (Pollak et al., 2002; Pollak et al., 2003b). However, not all therapies were equally effective in this model. Some anti-inflammatory treatments were ineffective unless combined, they often improved only some measures of sickness and depression and only dexamethasone, a general

inhibitor of cytokine production, had a mild influence on clinical scores. These therapies were far more successful at attenuating sickness behaviour after LPS injection. These observations indicate that not all anti-inflammatory therapies will be equally effective in EAE and that individual models of sickness behaviour and depression may operate according to different cytokine mechanisms (Pollak et al., 2003b).

In summary, we have demonstrated that treating EAE mice with the MAO inhibitor PLZ can be effective for improving behavioural deficits in mice with EAE. These effects relate to both the 'classical' locomotor deficits seen early in the disease course and other non-traditional measures of affect that relate to sickness and/or depression in the disease. Future studies can now be aimed at elucidating the specific pharmacological mechanisms of PLZ's effects in the disease to maximize these beneficial outcomes.

2.4 FIGURES AND TABLES

Figure 2.1

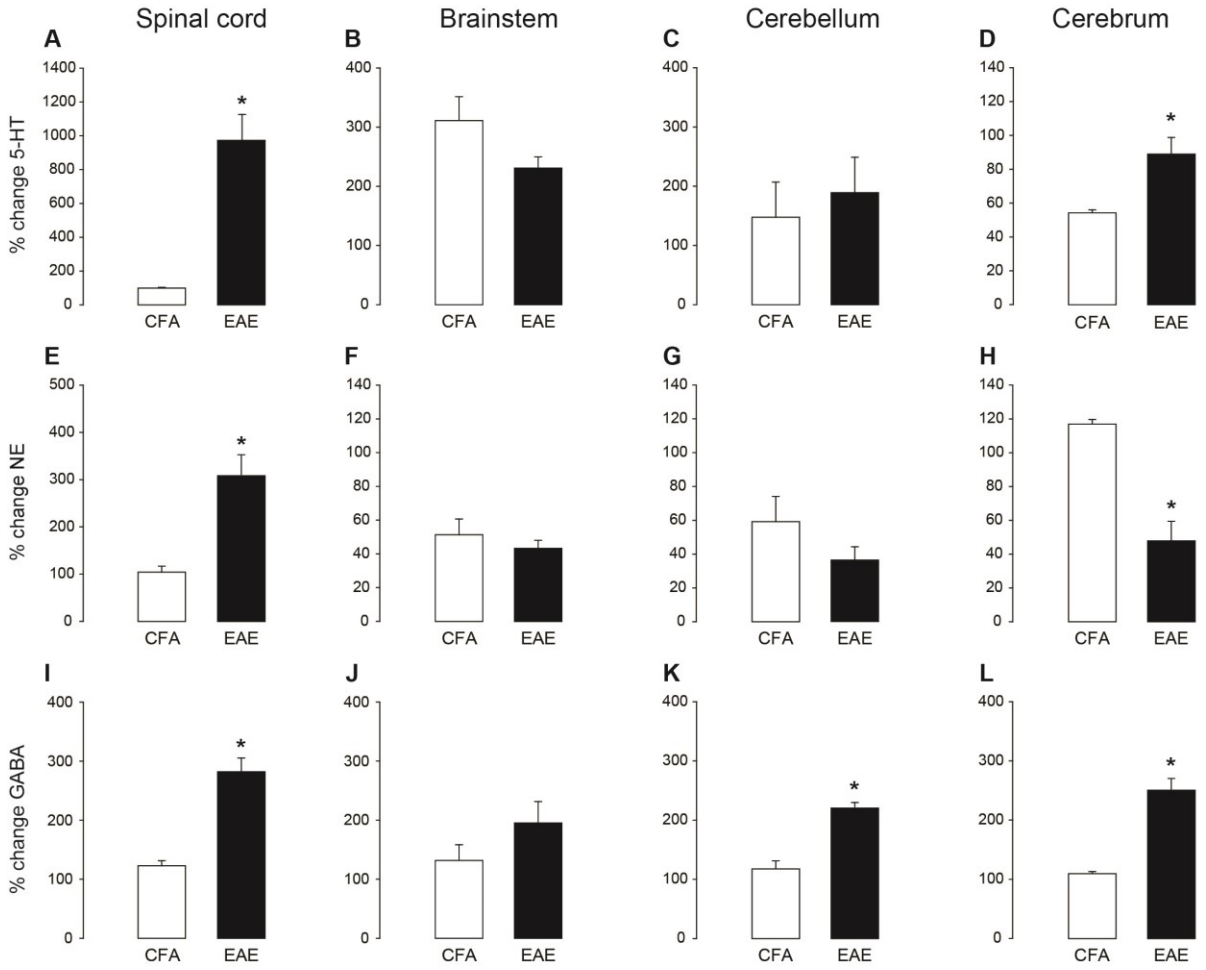

Figure 2.1: Acute treatment with PLZ increases the concentrations of 5-HT, NE and GABA in the CNS. (A-L) Normalized data relative to the concentrations of 5-HT, NE and GABA in untreated CFA controls from the spinal cord (A,E,I) brainstem (B,F,J) cerebellum (C,G,K) and cerebrum (D,H,L) of CFA and EAE mice treated with PLZ (30mg/kg, 3 hours prior to euthanizing). (A-D) PLZ-induced increases in 5-HT concentrations are significantly greater in the spinal cord (A) and cerebrum (D) of mice with EAE than in CFA controls but are similar in the brainstem and cerebellum (B,C). (E-H) PLZ-induced increases in NE levels are significantly greater in the spinal cord (E) of EAE mice than CFA mice but are less in the cerebrum (H) when

compared with CFA controls. PLZ causes a similar increase in NE levels in the brainstem and cerebellum in the two groups (F,G). (I-L) GABA concentration increases in the CNS of EAE and CFA mice after PLZ treatment are greater in the spinal cord (I), cerebellum (K) and cerebrum (L) during EAE in comparison to CFA controls. Values are the mean percent change from untreated CFA concentrations ± SEM. (* $P \leq 0.05$ Student's t-test).

Figure 2.2

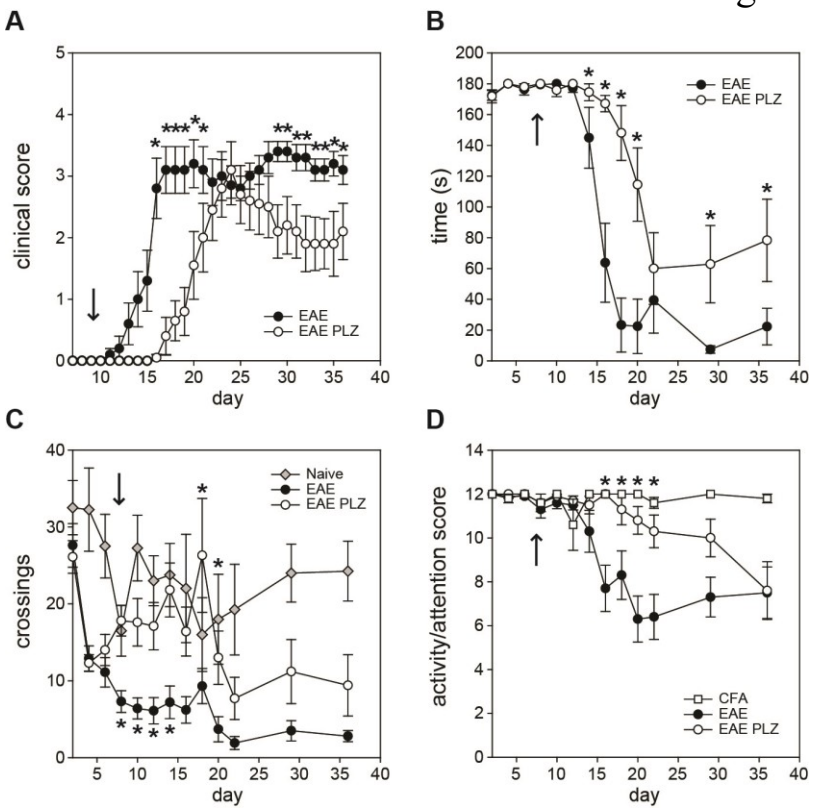

Figure 2.2: Daily PLZ treatment improves locomotor function and potentiates exploratory behaviours in mice with EAE. (A) PLZ treatment substantially delays the onset of clinical scores and reduces the impairments in the chronic phase of mice with EAE when compared with vehicle-treated animals. (B) Locomotor abilities on the rotorod are better maintained in EAE mice treated with PLZ than EAE mice receiving vehicle. (C) The number of open field crossings decreases in EAE mice compared to the amount performed by naive animals but is strongly increased for a limited time in EAE mice receiving PLZ. (D) The activity/attention scores of EAE mice treated with PLZ are maintained at control levels for a greater length of time compared to vehicle treated EAE mice. Values are mean ±SEM. (*P<0.05, Mann-Whitney Rank Sum test, EAE vs. EAE+PLZ). Arrow in A, B, C, D indicates point at which PLZ treatment began.

Figure 2.3

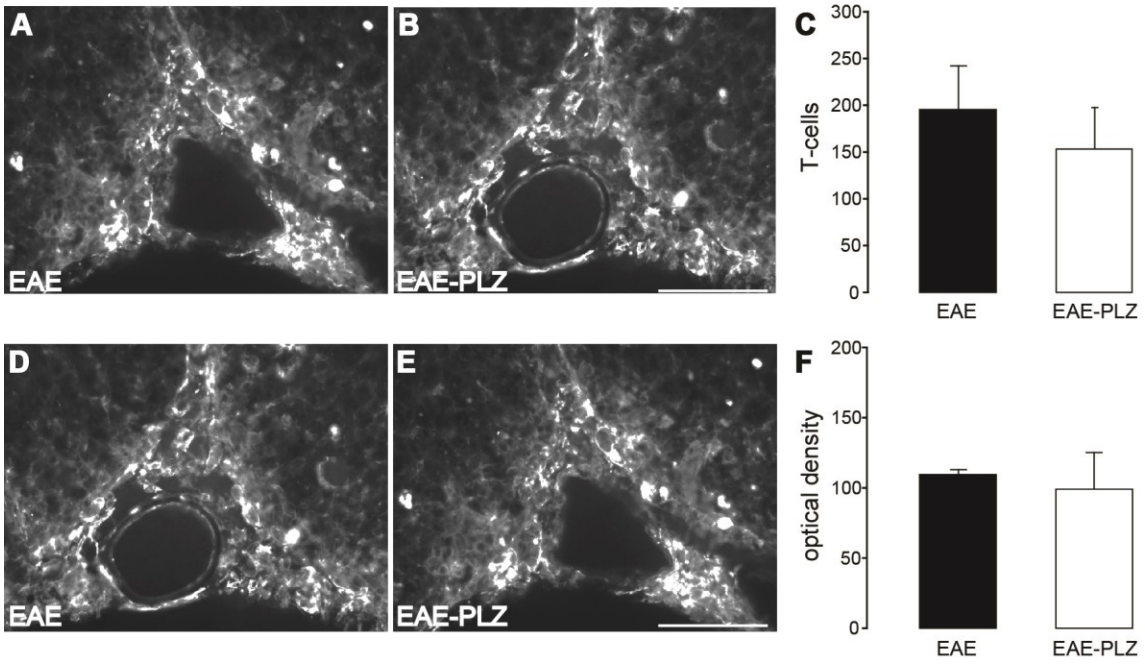

Figure 2.3: Daily PLZ treatment does not affect inflammation in mice with EAE. Cross sections of the ventral spinal cord immunostained for CD3+ T-cells (A,B) or Mac-1 (Cd11b) (D,E) reveal no significant differences between vehicle- and PLZ-treated mice with EAE (C,F). Scale bar in E=200μm and applies throughout.

Figure 2.4

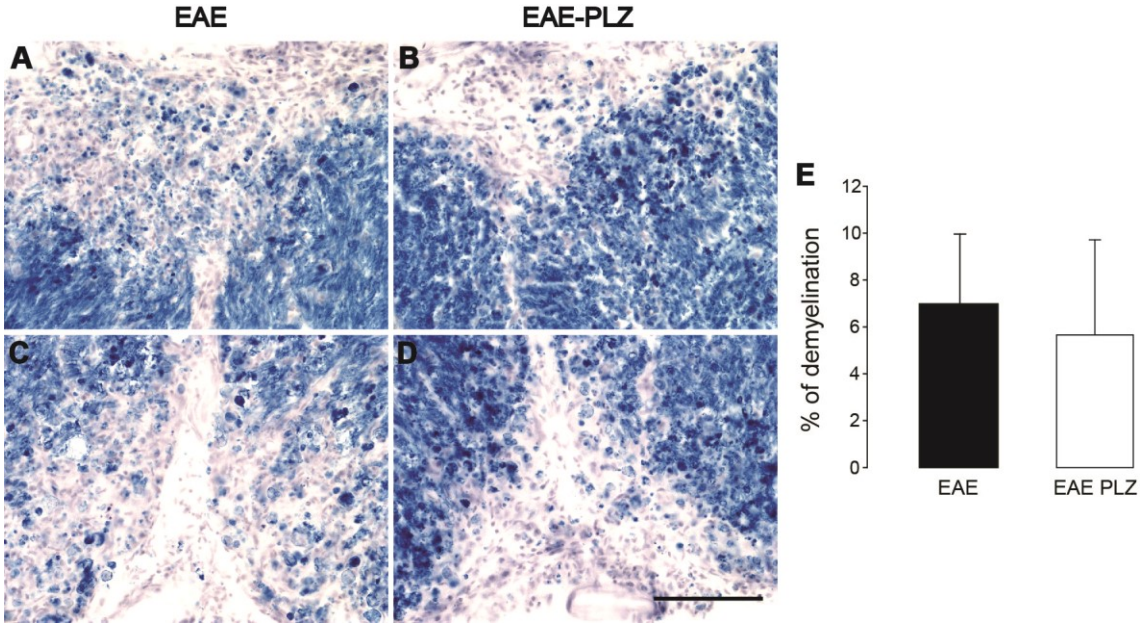

Figure 2.4: Daily PLZ treatment does not affect demyelination in mice with EAE. Eriochrome C-stained cross sections of the spinal cord centered on the dorsal columns (A,B) or ventral spinal cord (C,D) from vehicle (A,C) or PLZ treated (B,D) mice with EAE reveals no significant differences in the amount of demyelination between the two groups (E). Scale bar in D=200μm and applies throughout.

Figure 2.5

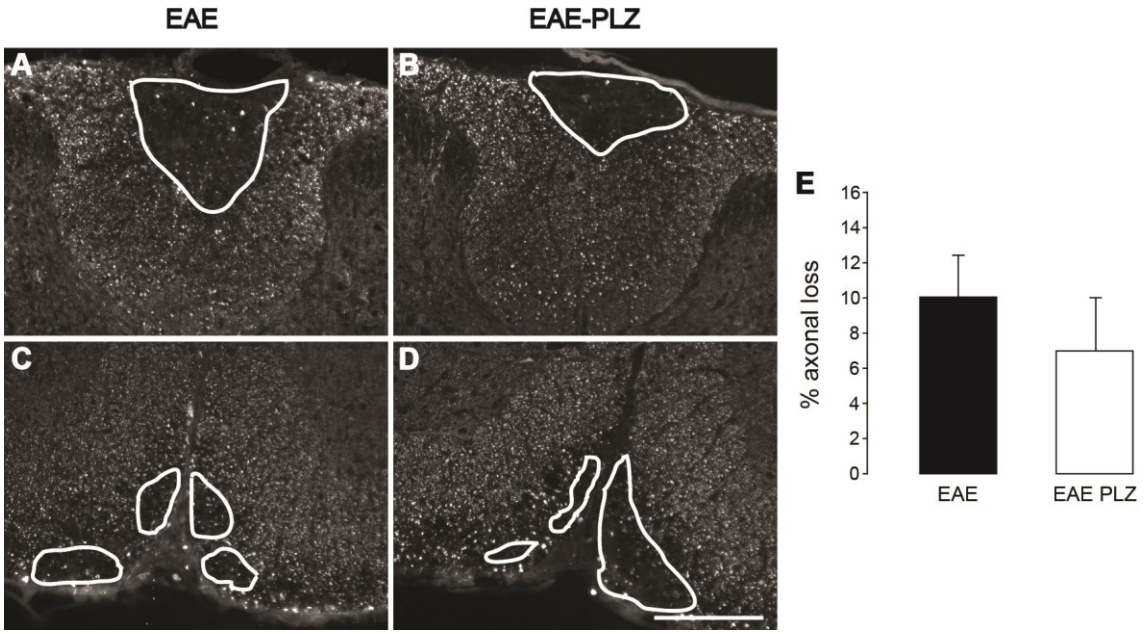

Figure 2.5: Daily PLZ treatment does not prevent axonal injury in mice with EAE. Cross sections of the spinal cord centered on the dorsal columns (A,B) or ventral spinal cord (C,D) from vehicle (A,C) or PLZ treated (B,D) mice with EAE stained for phosphorylated and non-phosphorylated neurofilament 200 (SMI-312 and SMI-32). PLZ- treated mice have a slight reduction in the amount of axonal loss but these differences do not reach statistical significance (E). Scale bar in D=200μm and applies throughout.

Figure 2.6

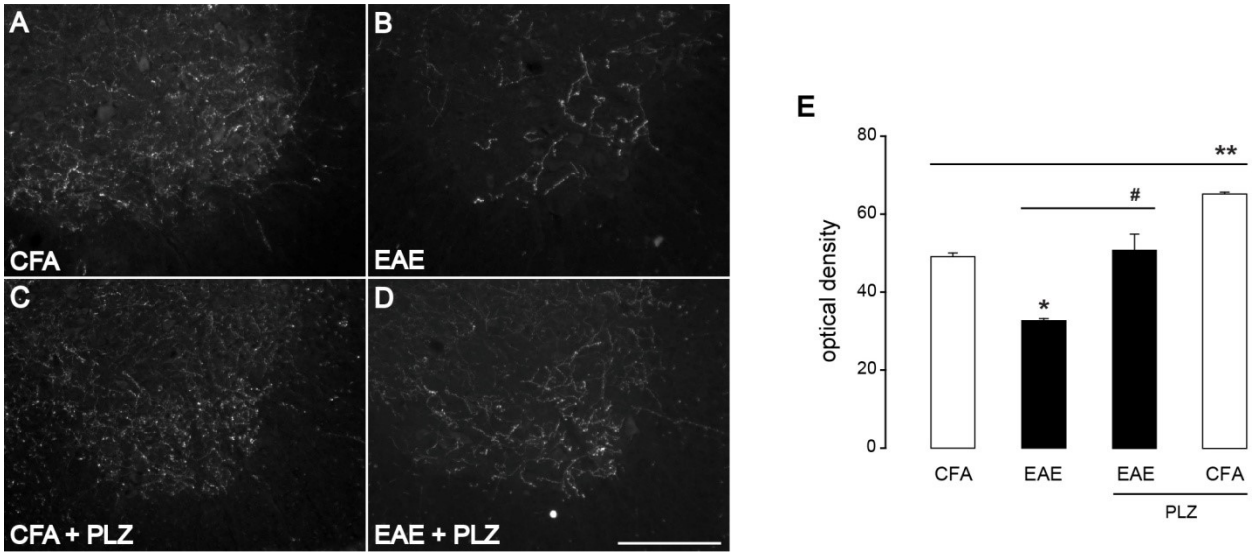

Figure 2.6: Daily PLZ treatment normalises 5-HT innervation patterns in the ventral horn of the spinal cord in mice with EAE. Cross sections of the spinal cord centered on the ventral horn from CFA (A), EAE (B), CFA+PLZ (C) and EAE+PLZ (D) immunostained for 5-HT. There is a significant reduction in 5-HT levels in vehicle-treated EAE mice (B,E). Daily PLZ treatment in EAE mice prevents this loss in 5-HT innervation (D,E). CFA controls treated with PLZ also show a significant elevation in 5-HT immunoreactivity compared to vehicle-treated CFA mice (C,E). (*P<0.05, one-way ANOVA, Tukey post hoc test vs. CFA; # P<0.05, one-way ANOVA, Tukey post hoc test vs. EAE; **P<0.05, one-way ANOVA, Tukey post hoc test vs. CFA). Scale bar in D=200μm and applies throughout.

Figure 2.7

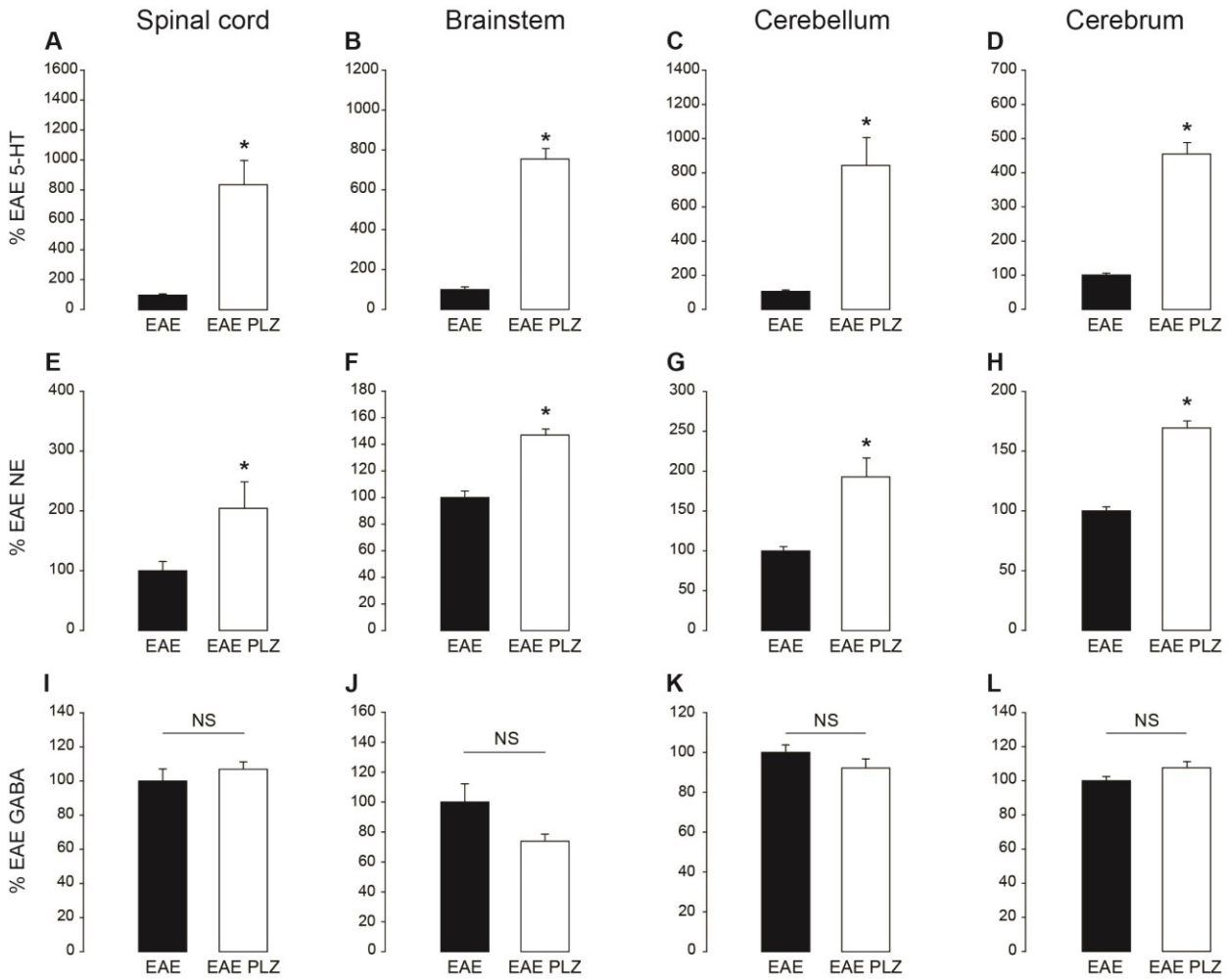

Figure 2.7: Chronic daily treatment with PLZ increases the concentrations of 5-HT and NE in the CNS but does not affect the levels of GABA in mice with EAE. Normalized data relative to the concentrations of 5-HT, NE and GABA in vehicle treated EAE mice from the spinal cord (A,E,I) brainstem (B,F,J) cerebellum (C,G,K) and cerebrum (D,H,L) after daily treatment with PLZ (15mg/kg/day). PLZ significantly elevates the levels of 5-HT and NE in all regions examined (A-H). However, daily PLZ does not change GABA levels relative to vehicle-treated EAE mice in any CNS region examined (I-L). Values are the mean percent of EAE levels from untreated EAE concentrations ± SEM. (* $P \leq 0.05$ Student's t-test).

Table 2.1

A

	Spinal cord				Brainstem			
	CFA	CFA PLZ	EAE	EAE PLZ	CFA	CFA PLZ	EAE	EAE PLZ
GABA	107.678	239.892	69.482	265.49	205.154	475.384	180.822	510.872
NE	242.4338	495.4026	76.178	311.7278	458.0274	691.7258	538.0926	770.6246
5-HT	778.632	1551.563	170.6566	1830.508	488.644	2009.399	779.2514	2577.424

B

	Cerebellum				Cerebrum			
	CFA	CFA PLZ	EAE	EAE PLZ	CFA	CFA PLZ	EAE	EAE PLZ
GABA	197.608	430.08	126.8	423.486	371.012	777.364	193.526	677.938
NE	252.787	402.334	289.106	394.367	342.936	743.723	371.886	549.857
5-HT	173.041	429.059	185.503	536.252	709.585	1094.559	753.946	1424.175

Table 2.1: Mean concentrations of GABA, 5-HT and NE in the CNS. Mean concentrations of GABA (μg/g tissue); 5-HT (ng/g tissue) and NE (ng/g tissue) from the spinal cord, brainstem (A) cerebellum, and cerebrum (rest of brain) (B) from CFA, CFA+PLZ, EAE and EAE+PLZ treated mice. These mice were euthanized 3 hours after treatment with PLZ or vehicle.

Table 2.2

	Spinal cord		Brainstem		Cerebellum		Cerebrum	
	EAE	EAE PLZ	EAE	EAE PLZ	EAE	EAE PLZ	EAE	EAE PLZ
GABA	89.68	92.76	190.70	146.41	195.00	172.65	287.13	286.66
NE	128.81	238.28	655.05	926.99	331.72	549.17	549.13	853.57
5-HT	132.26	722.82	432.56	2336.00	50.54	344.57	495.34	1826.82

Table 2.2: Mean concentrations of GABA, 5-HT and NE in the CNS of EAE mice after daily PLZ. Mean concentrations of GABA (μg/g tissue); 5-HT (ng/g tissue) and NE (ng/g tissue) from the spinal cord, brainstem, cerebellum and brain of EAE mice and EAE mice after chronic daily treatment with PLZ. These mice were euthanized 24 hours after the last daily treatment with PLZ or vehicle.

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Chapter 3

The MAO inhibitor phenelzine can improve functional outcomes in mice with established clinical signs in experimental autoimmune encephalomyelitis (EAE)

3.0 INTRODUCTION

Multiple sclerosis (MS) is a degenerative autoimmune disease of the central nervous system (CNS) characterized by acute inflammation, demyelination and neuronal degeneration (Compston and Coles, 2008). In addition, MS has been associated with reductions in the levels of key neurotransmitters (Barkhatova et al., 1998; Stover et al., 1997; White et al., 1983). Within the CNS of mice with experimental autoimmune encephalomyelitis (EAE), an animal model used to study MS, there are significant reductions in the amounts of norepinephrine (NE), 5-hydroxytryptamine (5-HT/serotonin) and γ-aminobutyric acid (GABA) (Musgrave et al., 2011b). Reductions in the levels of these neurotransmitters likely influence several disease mechanisms of MS.

Many neurotransmitters are capable of directly influencing and driving immune cell function. T-cells and macrophages express the receptors to respond to and the machinery to synthesize 5-HT, glutamate, GABA, and dopamine (DA) (Besser et al., 2005; Bhat et al., 2010; Sarchielli et al., 2007a; Yin et al., 2006). Exposure of immune cells to these neurotransmitters can occur both within the CNS and in the periphery (Levite, 2008). Therefore, pharmacological agents that modulate the levels of these neurotransmitters could act on T-cells prior to or after their migration into the CNS. In EAE, multiple studies have indicated the effectiveness of both GABA and monoamines (5-HT, NE, DA) in reducing disease severity (Bhat et al., 2010; Hofstetter et al., 2005; Simonini et al., 2010).

In addition to the well-described motor impairments, MS is also associated with significant neurological disabilities such as fatigue, depression, cognitive deficits and neuropathic pain (Compston and Coles, 2008). Similarly, mice with EAE also display behavioral

signs of depression, anxiety and enhanced pain sensitivity (Olechowski et al., 2009; Peruga et al., 2011; Pollak et al., 2002). Changes in the amounts of neurotransmitters have been implicated in these non-motor symptoms. Damage to the locus coeruleus, the primary CNS source of NE, has been observed in both MS and EAE (Polak et al., 2011). The locus coeruleus has important roles in the control of attention, memory and arousal (Samuels and Szabadi, 2008). In addition, reductions in the levels of GABA have been implicated in depression and anxiety disorders (Lydiard, 2003). Pharmacological recovery of NE and GABA could therefore, improve these behaviors in EAE.

PLZ is a non-selective, irreversible inhibitor of monoamine oxidase (MAO) that causes long lasting increases in the levels of biogenic amines (Parent et al., 2000). Clinically, PLZ has been used to treat major depression, panic disorder and social anxiety disorder (Gitow, 1994; Johnson, 1994; Kennedy, 2009) and it has been reported to have neuroprotective properties in an animal model of ischemia-reperfusion brain injury (Wood et al., 2006b). Uniquely, PLZ is also a substrate for the MAO enzyme (Clineschmidt and Horita, 1969a, c; Popov and Matthies, 1969a) and a product of this interaction is β-phenylethylidenehydrazine (PEH) (MacKenzie et al., 2010b). PEH is a potent inhibitor of GABA transaminase, which leads to surges in GABA levels in the brain (Baker et al., 2007; Paslawski et al., 2001). We have previously demonstrated that daily PLZ treatment starting at day 7 post EAE induction delayed the onset of EAE symptoms and reduced the severity of disease (Musgrave et al., 2011a).

In the present study we set out to determine whether beginning PLZ treatment in an established disease state could improve behavioral outcomes. We show that in this more clinically relevant paradigm that PLZ treatment can significantly reduce disease severity and is effective at improving motor and non-motor behavioral outcomes.

3.1 METHODS

3.1.1 EAE Induction.

A total of 35, 10 to 12 week old female C57/BL6 mice (Charles River) were used in these experiments. EAE was generated using myelin oligodendrocytes glycoprotein 35-55 (MOG_{35-55}) obtained from the Peptide Synthesis Facility at the University of Calgary and following a protocol approved by the local animal ethics committee. EAE was induced by subcutaneous injection of 50 µg of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 2.5 mg/mL. An intraperitoneal injection of 300 ng pertussis toxin (List Biological Labs, Campbell, CA, USA) was administered on the day of induction and 48 hours later in EAE immunized mice. Only mice that went on to develop clinical signs of the disease were included for subsequent behavioral, histological or HPLC analysis.

3.1.2 EAE assessment

Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1 (disease onset), flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both (Musgrave et al., 2011a; Olechowski et al., 2009)

3.1.3 Drug Treatment

Cohorts of mice were treated on alternating days with the MAO inhibitor phenelzine (PLZ)(15mg/kg i.p) (Sigma) beginning when a mouse exhibited the first clinical signs of EAE (clinical grade 1, variable from day 10-14 after induction). When a mouse reached clinical grade

1 they were then treated with PLZ (n=14) or vehicle (saline, n=12) intraperitoneally every second day for 14 days (7 injections total). Starting PLZ administration only at the onset of clinical signs in each subject represents a more clinically relevant treatment approach compared to our previous experiment where PLZ treatment began at 7 days post induction, prior to the onset of any clinical signs in all mice (Musgrave et al., 2011a). All treatments were administered by an experimenter blinded to the experimental conditions. An additional experiment was carried out to determine the level of MAO inhibition that occurs with PLZ treatment. An additional group of mice (n=5) was induced with EAE (as above) and once clinical signs of the disease within a given mouse became evident, daily treatment with PLZ began for 14 consecutive days (14 injections total).

3.1.4 Open field assays

Open field analysis was conducted as previously described (Musgrave et al., 2011a). Mice are placed in an open field (width: 29 cm, length: 44 cm, height: 17 cm) and observed for a period of 4 minutes. As a measure of exploratory behavior the total number of crossings into each adjacent quadrant of the open field was recorded. A general activity/attention score was also calculated for the 4-minute observation period. The Activity/Attention Score represents the amount of time a mouse spent in a specific pre-defined sedentary posture (Musgrave et al., 2011a; Olechowski et al., 2009). This posture is defined as having both forepaws on the ground and with the head being still with a steady gaze directed below the horizontal (i.e. a head position with the nose pointing toward the floor of the observation box). These postures are never observed in naive animals and only rarely in control mice immunized with CFA alone. The Activity/Attention Score is based on a 4-point scale that is assigned for each minute of the observation period

totaling a maximum of 12 points. Mice are scored based on the following criteria: 0, mouse is still and with a floor-directed gaze for 45 or more seconds in one minute; 1, mouse is still and with a floor-directed gaze for 30 to 45 seconds in one minute; 2, mouse is still and with a floor-directed gaze for 15 to 30 seconds in one minute; 3, mouse is still and with a floor-directed gaze for 0 to 15 seconds in one minute.

3.1.5 Rotorod assay

As a test of gross locomotor ability, mice were tested on a 14-rpm, fixed speed rotorod. The duration the mice were able to remain on the rotorod was recorded up to a maximum of 180 sec. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. Each mouse had three trials and the mean latency to fall per trial was calculated.

3.1.6 HPLC

HPLC was carried out on tissue (spinal cord, brainstem, whole brain) from a subset of the mice treated with vehicle or PLZ (n=5, EAE+ vehicle; n=5, EAE+PLZ). The mobile phase solutions were all made using HPLC grade methanol (MeOH), tetrahydrofuran (THF), and acetonitrile purchased from Fisher Scientific. Standards and tissue samples were analyzed for the levels of amino acids using a minor modification of the procedure of Grant et al. (2006). Tissues were homogenized in 5 volumes of ice-cold MeOH, left on ice for 10 minutes and then centrifuged at 10,000xg for 4 minutes. The resultant supernatants were then diluted in water to make a 30- or a 60-fold dilution. Portions of these diluted supernatants were then reacted with *o*-pthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBC) and the resultant derivatives used for analysis, with

the fluorescence detector set at an excitation wavelength of 344 nm and an emission wavelength of 433 nm. Standardization was established based on calibration curves prepared from authentic samples of amino acids that were generated for each individual run of samples. The procedure of Parent et al. (2001) was used for the separation and analysis of biogenic amines and their metabolites. To aliquots of tissue in 5 volumes of water, $1/10^{th}$ the volume of ice-cold 1N $HClO_4$ containing ascorbic acid (500μM) and EDTA (100 mg %) was added. These homogenates were vortexed, centrifuged at 10,000xg for 4 minutes and the supernatant was removed for analysis by HPLC. The applied potential for electrochemical detection was 0.65 V. Calibration curves were constructed for each HPLC run.

3.1.7 Histology and Immunocytochemistry

Immunocytochemistry was carried out on spinal cord tissue from various levels (lumbar, thoracic, cervical) from a subset of the mice treated with vehicle or PLZ (n=5, EAE+ vehicle; n=5, EAE+PLZ). At the end of the treatment period (14 days after disease 'onset') mice were anesthetized and sacrificed by transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Lumbar, thoracic and cervical regions of the spinal cord were removed, post-fixed overnight and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm). To assess inflammation and reactive gliosis, a rat anti-CD4 (1:200, Serotec) and rat anti-IBA1 (1:750, Wako) were used. Changes in the levels of 5-HT expression in the spinal cord were assessed using a rabbit anti-5-HT (1:5000, Sigma) antibody. These were visualized using goat anti-rat or anti-rabbit Alexa Fluor®594 or 488 (1:200, Molecular Probes, Eugene, OR).

3.1.8 Quantification of histology and immunocytochemistry

Images were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All image analysis was carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. The number of CD4+ cells within the parenchyma of the ventral spinal cord was counted in sections from the lumbar, thoracic and cervical spinal cord. An average number of CD4+ T-cells was calculated per section (3 sections per slide, 2 slides per animal, n=5 animals per group). Optical density measurements of IBA1 immunoreactivity were examined in the same region of the ventral spinal cord (3 sections per slide, 2 slides per animal, n=5 animals per group). The level of background staining was determined for each section and subtracted for all optical density measurements. Using the same optical density technique, the innervation density of 5-HT was assessed in cross-sections of a standardized ventral horn area of the lumbar, thoracic and cervical spinal cord (3 sections per slide, two slides per animal, analyzing both ventral horns per section, n=5 animals per group). Staining density was measured using NIH ImageJ software using a manual threshold function to quantify the optical density of staining within the standardized area of ventral horn from each section.

3.1.9 MAO Assay

Brain and spinal cord tissue homogenate was generated from EAE mice treated with either VEH, 7 doses or 14 doses of PLZ. The tissue homogenate was diluted in potassium phosphate buffer (0.2 M, pH 7.4) and 50µl was incubated with 50µl of the appropriate radiolabeled substrate (^{14}C-labeled 5-hydroxytryptamine was used for MAO-A and β-phenylethylamine was used for MAO-

B). Each sample tube was flushed with gaseous oxygen and incubated at 37°C for 10 minutes. The reaction was stopped by adding 10µl of HCL (3M) to each sample. The reaction products were extracted using 1ml of a 1:1 mixture of ethyl acetate and toluene and centrifuged for 30 seconds. Next, a 700µl aliquot of the upper layer was removed and added to a vial containing scintillation fluid. The radioactivity was counted Beckman LS 7500 liquid scintillation spectrometer.

3.1.10 Statistical analysis

Statistical analysis was carried out using the Student's t-test and one-way ANOVA with Tukey post hoc tests. For non-parametric data the Mann-Whitney Rank Sum test was used. Significance was set at $P < 0.05$.

3.2 RESULTS

3.2.1 Functional improvement with PLZ treatment in established EAE

Previously, we investigated the effects of chronic PLZ treatment starting at 7 days post EAE induction. We observed significant improvements in clinical score and in behavioral measures of anxiety and depression early in the disease course (Musgrave et al., 2011a). We therefore decided to assess the effects of PLZ treatment using a more clinically relevant treatment paradigm. In the current study, each mouse with EAE began treatment with PLZ when the first signs of neurological deficit (clinical grade 1) appeared. PLZ was administered on alternating days over the course of a 14-day observation period.

Treating EAE mice with this protocol significantly improved clinical signs and disease progression (Fig. 3.1A). While EAE mice treated with PLZ reached a similar peak clinical grade compared to vehicle-treated mice, the progression of disease in PLZ-treated animals was slower

and was accompanied by better functional recovery in these mice (Fig. 3.1A). As an adjunct measure to the evaluation of clinical signs in EAE, gross locomotor ability was also assessed using a rotorod assay. We find that PLZ treatment significantly improves motor performance compared to vehicle treated controls after 6 days of treatment (3 injections) (Fig. 3.1B) and these improvements were sustained (last measurement at 12 days on treatment, 6 injections total) (Fig. 3.1C).

PLZ treatment also leads to significant improvements in open field behaviors. Exploratory behavior, as measured by the number of line crossings in an open field, decreases dramatically prior to the onset of EAE symptoms (data not shown). However, by the second treatment day after disease onset, EAE mice treated with PLZ show significantly greater amounts of line crossings in an open field (Fig. 3.1D). In addition, using the composite activity/attention score, based on each mouse's activity levels and attentiveness to the surrounding environment (Musgrave et al., 2011a), we found that vehicle-treated EAE mice continuously decline in this measure over the 14 treatment days (Fig. 3.1E). In contrast, PLZ-treated animals did not display the same decreases in the activity/attention score. The activity/attention scores of PLZ-treated mice were near normal baseline levels by the end of the 14-day observation period (Fig. 3.1E).

3.2.2 PLZ does not change the amount of T-cell infiltration or microglial activation within the spinal cord in established EAE

To determine whether PLZ treatment influenced the amount of cellular pathology within the CNS, we examined the lumbar, cervical and thoracic regions of the spinal cord for the number of CD4+ T-cell infiltrates and the levels of Iba-1 reactivity; a marker for microglia/macrophages from tissue taken at the end of the 14 day treatment period. We observed no significant

difference in the number of infiltrating CD4+ T-cells between the PLZ- and vehicle-treated groups in any region of the spinal cord (Fig. 3.2A and B). Similarly, PLZ treatment did not have a significant influence on the density of Iba-1 staining in spinal cord sections from the lumbar, cervical and thoracic levels (Fig. 3.3A and B).

3.2.3 PLZ increases 5-HT innervation throughout the spinal cord in established EAE

Mice with EAE have a significant reduction in the levels of 5-HT within the ventral horn of the spinal cord. We have shown previously that PLZ treatment prior to disease onset can normalize 5-HT levels within the ventral horn of the spinal cord in mice with EAE (Musgrave et al., 2011a). We therefore assessed whether starting PLZ treatment after the onset symptoms could have a similar effect. We examined 5-HT levels in the ventral horn from lumbar, thoracic and cervical sections of spinal cord at the end of the 14-day treatment period. Despite beginning treatment after the disease has been established we found that PLZ is still effective at elevating the levels of 5-HT in the ventral horn of EAE mice throughout the spinal cord (lumbar, thoracic and cervical levels) compared to vehicle-treated controls (Fig. 3.4A and B).

3.2.4 PLZ modifies the levels of amino acids and biogenic amines throughout the CNS in established EAE

After the onset of clinical signs and the subsequent 14-day treatment period, we also conducted HPLC analysis on spinal cord, brain and brainstem tissue samples from EAE mice treated with PLZ or vehicle at disease onset to examine the levels of the amino acids glutamate, glycine, D-serine and GABA. We have previously shown that the levels of glycine and D-serine are increased with progressive EAE. While the levels of GABA and glutamate are decreased in

chronic EAE when analyzed using HPLC (Musgrave et al., 2011b). In EAE mice that received PLZ treatment after disease onset there was a significant decrease in the levels of glycine in the brain compared to vehicle treated EAE mice (Fig. 3.5 F). In addition, PLZ treatment caused a general decrease in the levels of D-serine in the spinal cord, brainstem and brain samples (Fig. 3.5 G-I). PLZ treatment did not alter the amount of glutamate within the CNS (Fig. 3.5 A-C). However, GABA levels were similarly increased in the spinal cord and brain of PLZ treated mice compared to EAE mice treated with vehicle (Fig. 3.5 J-L).

In addition, HPLC was also used to examine the levels of the biogenic amines within the spinal cord, brain stem and brain. Previous experiments have shown that the levels of 5-HT, NE, DA are all significantly diminished in EAE mice compared to mice treated with CFA alone (Musgrave et al., 2011a). EAE mice that receive PLZ treatment after disease onset had substantial increases in 5-HT and NE levels in the spinal cord, whole brain, and brainstem compared to vehicle-treated EAE mice (Fig. 3.5A-F). In addition, PLZ treated mice had significantly greater levels of DA in the spinal cord and brain compared to vehicle treated EAE mice (Fig. 3.5G-I). Based on observations from our previous experiments, we can conclude that the levels of these neurotransmitters/neuromodulators are effectively normalized with PLZ treatment in established EAE.

3.2.5 Reduced MAO inhibition with alternating vs. daily PLZ treatment

Previously we have shown that chronic daily administration of PLZ does not sustain significant increases in GABA levels in the CNS. We hypothesized that this inability to maintain elevated levels of GABA was due to excessive inhibition of the MAO enzyme by prolonged exposure to PLZ. PLZ's ability to elevate GABA levels is dependent on MAO activity (Popov and Matthies,

1969a; Todd and Baker, 1995). We therefore assessed the amount of MAO inhibition that is produced by PLZ treatment in mice that were treated daily for 14 days (14 doses) compared to mice treated on alternating days for 14 days (7 doses) used for the current behavioral observations. Both the daily (14 doses) and alternating (7 doses) administration of PLZ was highly effective at inhibiting MAO enzyme function (MAO A and MAO B) (Fig. 3.6 A, B). Inhibition of the MAO A enzyme was close to 95% in both brain and spinal cord samples and showed no difference between the 'alternating' 7 doses compared to the 'daily'14 doses of PLZ. However, alternating PLZ doses produced statistically less inhibition of MAO B compared to 14 injections in both the brain and spinal cord of EAE mice. In the brain, 14 doses of PLZ lead to a 95.7% inhibition of MAO B compared to 92.2% inhibition with 'alternating' 7 doses (Fig. 3.6A, right panel). Within the spinal cord, 14 doses of PLZ were very effective reaching 99.5% MAO B inhibition, whereas 7 doses produced a slight but significant reduction of MAO B inhibition (Fig. 3.6B, right panel). These modest but significant differences in the amount of MAO B inhibition might account for the sustained increases in GABA levels that we find here when PLZ is administered on alternating days.

3.3 DISCUSSION

We have previously shown that treating mice with EAE prior to the onset of symptoms, with the MAO inhibitor PLZ is able to reduce the severity of clinical signs in the disease. In our current study we demonstrate that PLZ, when given after the clinical signs of EAE have become established, is still effective and can sustain the improvements in both motor and non-motor outcomes in mice with EAE. These behavioral improvements in PLZ-treated mice were associated with decreased levels of glycine and D-ser and significantly greater levels of GABA,

and biogenic amines (5-HT, NE, DA) in the brain and spinal cord as well as increased levels of 5-HT and NE throughout the neuraxis. Furthermore, we find that PLZ treatment increased the density of 5-HT innervation in the ventral horn of the spinal cord which is a likely mechanism leading to the improved motor outcomes in PLZ-treated mice.

In our earlier studies we observed that when we began treating mice with EAE prior to the onset of clinical signs that the beneficial effects of daily PLZ did not lead to lasting improvement in behavioral outcomes. In fact when PLZ was administered for such a prolonged period the beneficial effects of PLZ treatment diminished by the end of the experiment (Musgrave et al., 2011a). We hypothesized that the failure of daily PLZ to sustain improvements in EAE disease progression was due to a transient suppression of the immune mechanisms involved in EAE. We predicted that this transient effect was caused by the inability of chronic daily PLZ to maintain an increase in GABA levels over the course of the experiment (Musgrave et al., 2011a). There is strong evidence that agents that can elevate GABAergic signaling are effective at reducing the severity of EAE (Bhat et al., 2010; Hofstetter et al., 2005; Simonini et al., 2010). The inability to sustain elevations in GABA with long term PLZ treatment is related to the potency of PLZ's inhibition of MAO. PLZ is an irreversible inhibitor of MAO that leads to increases in the levels of biogenic amines (5-HT, NE and DA). However, it is also a substrate for MAO that leads to the production of the metabolite PEH (Al-Nuaimi et al., 2012). PEH is a potent inhibitor of GABA transaminase and it is the production of the PEH metabolite from PLZ that leads to the large increases in GABA levels. Therefore, the production of PEH and subsequent increase in GABA levels is dependent on having active MAO enzymes that have not bound PLZ. We find that chronic daily PLZ treatment causes a substantial decrease in MAO activity (McKenna et al., 1991) that would lead to a reduced production of PEH. The lack of

PEH production with ongoing PLZ treatment would result in a loss of GABA transaminase inhibition and GABA levels would return to those seen in untreated EAE mice. Therefore, a treatment schedule that produces less MAO inhibition would maintain PEH levels and sustain high amounts of GABA. To validate the new treatment protocol used here, we compared the amount of MAO inhibition between alternating and daily PLZ treatment. Alternating the PLZ treatment day was still highly effective at inhibiting MAO, but importantly the level of inhibition was less than that observed with the daily treatment. Although the difference in MAO inhibition between treatment schedules was small, PLZ treatment on alternating days led to significant GABA elevations that persisted over the two-week testing period.

Multiple studies have associated ventral spinal cord 5-HT innervation with motor activity. Modulation of ventral horn 5-HT has been linked to locomotor function, and exogenous 5-HT improves locomotion in models of spinal cord injury (Hayashi et al., 2010; Zhou and Goshgarian, 2000). In MS, a study examining CSF neurotransmitter and metabolite levels showed the greatest correlation between reduced 5-HT levels and disease severity (Markianos et al., 2009). These findings demonstrate that a reduction in 5-HT levels has a significant role in MS pathology. Previously, we demonstrated that starting PLZ treatment prior to the onset of clinical signs normalized the innervation pattern of 5-HT in the ventral horn of the spinal cord (Musgrave et al., 2011a) . We have now assessed PLZ treatment in mice with established EAE and find the same significant elevation in the amount of serotonergic fiber density within the ventral horn of the spinal cord. This suggests that PLZ, possibly through increases in 5-HT, is able to maintain neuronal excitability in locomotor circuits that may be an important mechanism for the observed functional recovery in PLZ-treated EAE mice.

We have previously shown that EAE animals have increased levels of glycine and D-ser

within the CNS (Musgrave et al., 2011b). Glycine can be both inhibitory and excitatory. Activation of the glycine receptors causes fast inhibitory signaling; however glycine is also a co-agonist for the excitatory NMDA receptor. In addition, glycine has been shown to have anti-inflammatory properties in models of peripheral immune activation (Zhong et al., 2003). However, it has also been shown that glycine can increase the uptake of myelin and TNF-α production by peritoneal macrophages (Carmans et al., 2010). Although in our experiment there was no difference in macrophage activation, our technique may not be sensitive enough to detect glycine induced changes.

D-Ser is a co-agonist for the NMDA receptor and increased D-ser is associated with excitotoxicity. In particular, in the neuroinflammatory disease amyotrophic lateral sclerosis (ALS) there is an up regulation of D-Ser and its biosynthesis enzyme, serine racemase that contributes to the NMDA receptor induced excitotoxic damage in ALS (Sasabe et al., 2007). PLZ treatment reduced D-Ser levels in EAE towards normal levels and may limit the damage caused by the abundance of excitatory signals.

Phenelzine is also very effective at sequestering reactive aldehydes, including acrolein (Wood et al., 2006b), an aldehyde that has been proposed to be present in excess in MS, leading to oxidative stress (Shi et al., 2011). These latter researchers have suggested that hydrazine-containing drugs such as hydralazine may be effective treatments for MS, and phenelzine would fall into this category.

GABA, 5-HT, NE and DA have each been shown to reduce the severity of EAE disease through anti- inflammatory mechanisms (Bhat et al., 2010; Hofstetter et al., 2005; Levite, 2008; Simonini et al., 2010). Lymphocytes express the receptors needed to respond to GABA, 5-HT, NE, DA. Both T-cells and macrophages express functional GABA-A receptors although the

expression of specific GABA-A receptor subtypes is species dependent (Bhat et al., 2010; Mendu et al., 2012). 5-HT can modulate the proliferation of T-cells and induce cytokine release from monocytes through the binding to different 5-HT receptor subtypes (Durk et al., 2005; Yin et al., 2006). T-cells can also respond to NE through the alpha and beta2-adrengic receptors (McAlees et al., 2011), although the effect of receptor activation is dependent on the T-cell phenotype and the cytokines that are present (Sanders, 2012). Similarly, T-cells express functional D2, D3, D4 and D5 receptors (McKenna et al., 2002). The binding of DA to these receptors can suppress the function of activated T-cells (Levite, 2008). Thus, sustained high concentrations of GABA, 5-HT, NE and DA in response to PLZ could modulate the function of the myelin reactive T-cells within the CNS without preventing their entry.

In addition, PLZ could have a direct effect on immune cell function. Human lymphocytes express MAO-A on the mitochondrial membrane (Chaitidis et al., 2004). Furthermore, the Th-2 cytokines IL-4 and IL-13 cause an upregulation of MAO-A, indicating that MAO-A may be involved in immune regulation (Chaitidis et al., 2004). Therefore, PLZ could be modulating lymphocytes by inhibiting MAO. The presence of MAO in lymphocytes would allow for the production of the GABA-T inhibitor PEH with PLZ treatment. This would lead to surges in the levels of GABA in immune cells. Both macrophages and T-cells contain the enzyme GABA-T (Bhat et al., 2010) and it has recently been shown that treating macrophages with two irreversible GABA-T inhibitors, vigabatrin and gabaculine, reduced IL-1β and IL-6 expression (Bhat et al., 2010).

In summary, we have demonstrated that treating mice with established EAE with the MAO inhibitor PLZ leads to significant elevations in the levels of GABA and the biogenic amines 5-HT, NE and DA in the CNS. This is accompanied by significant improvements in a

number of behavioral outcome measures. Combined with our previous work, these results indicate that the increases in GABA and biogenic amine levels are critical factors in limiting clinical signs in EAE.

3.4 FIGURES

Figure 3.1

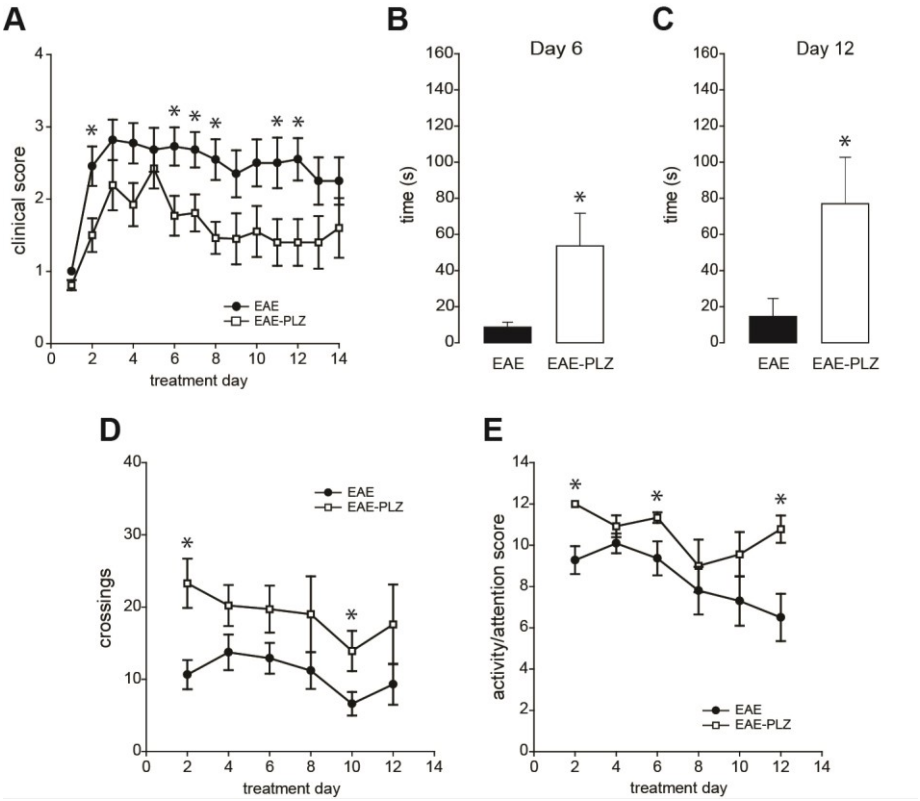

Figure 3.1: PLZ treatment in established EAE improves clinical signs and locomotor function in EAE.

(A) PLZ treatment starting at the onset of clinical signs (clinical grade 1) maintains lower clinical disease scores over the course of the two-week observation period. (B, C) Gross locomotor function is significantly better in mice receiving PLZ treatment at day 6 after beginning treatment (3 doses) (B) that is maintained at 12 days after the beginning of treatment (6 doses) (C). (D) The number of line crossings in an open field is significantly increased in EAE mice receiving PLZ compared to vehicle-treated animals. (E) Beginning PLZ treatment in established EAE leads to significantly higher activity/attention scores. (* $P<0.05$, Mann-Whitney Analysis, A, D, E) (* $P<0.05$ t-test, C, D). Error bars in all panels represent SEM.

Figure 3.2

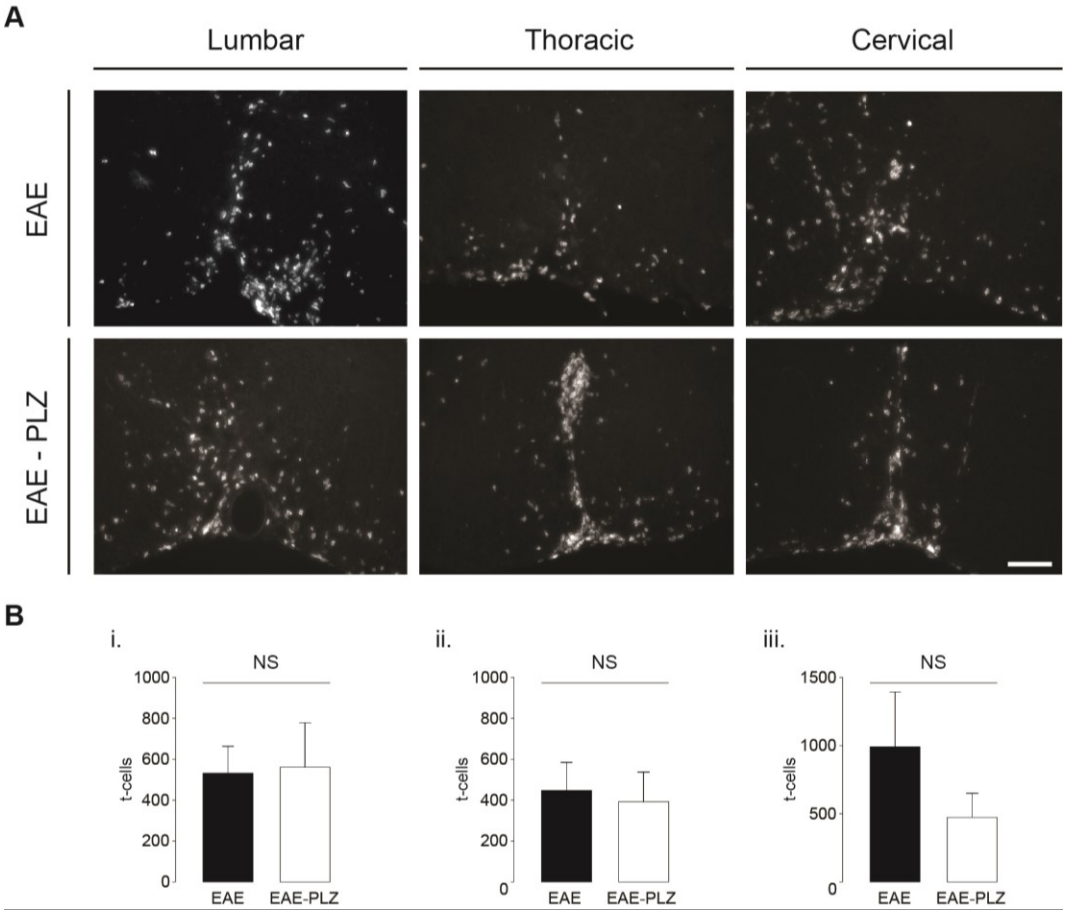

Figure 3.2: PLZ treatment in established EAE does not affect T-cell infiltration in mice with EAE. (A, B) Cross sections of i) lumbar, ii) thoracic and iii) cervical ventral spinal cords immunostained for CD4+ T-cells revealed no significant differences between vehicle- and PLZ-treated mice with EAE. (NS $P>0.05$) Scale bar = 100 μM and applies throughout. Error bars in (B) panels i, ii, iii, represent SEM.

Figure 3.3

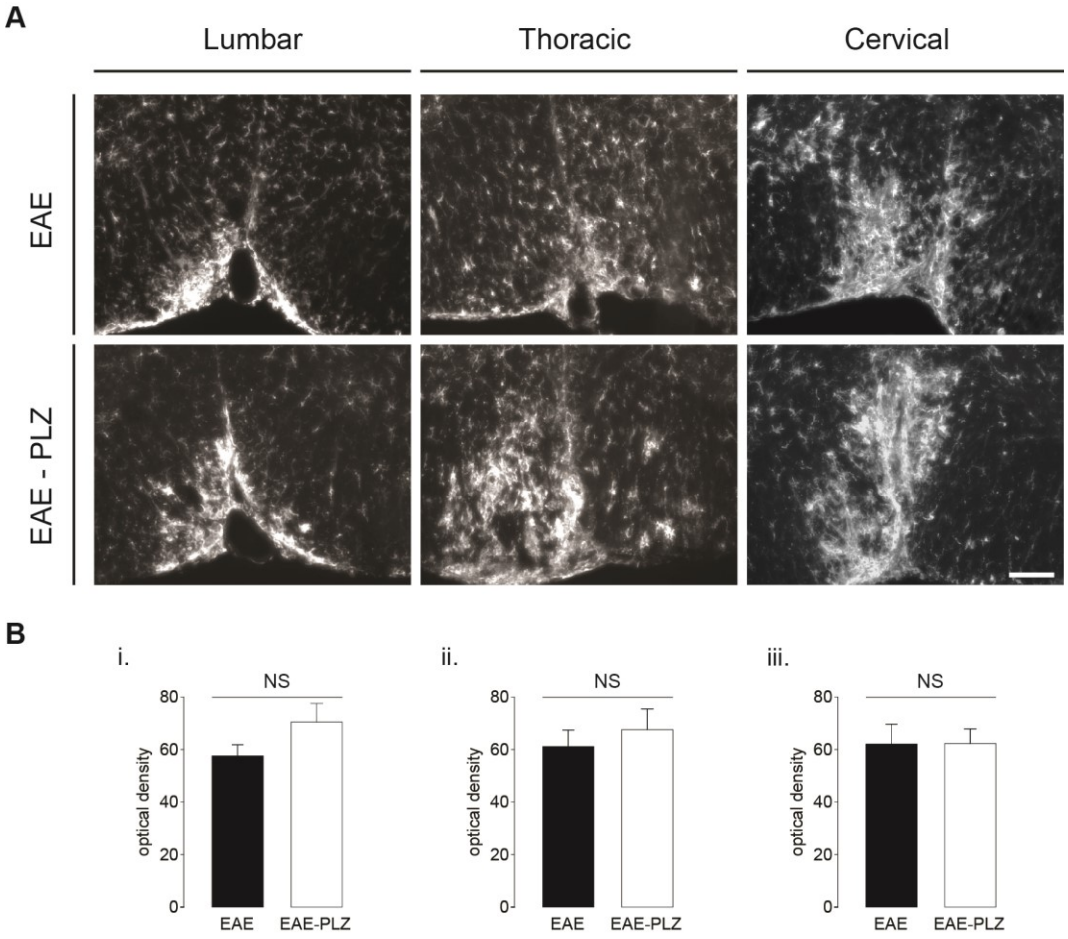

Figure 3.3: PLZ treatment in established EAE does not affect microglia/macrophage reactivity in mice with EAE. (A, B) Cross sections of i) lumbar, ii) thoracic and iii) cervical ventral spinal cords immunostained for Iba-1 revealed no significant differences between vehicle- and PLZ-treated mice with EAE. (NS $P>0.05$) Scale bar = 100 μM and applies throughout. Error bars in (B) panels i, ii, iii, represent SEM.

Figure 3.4

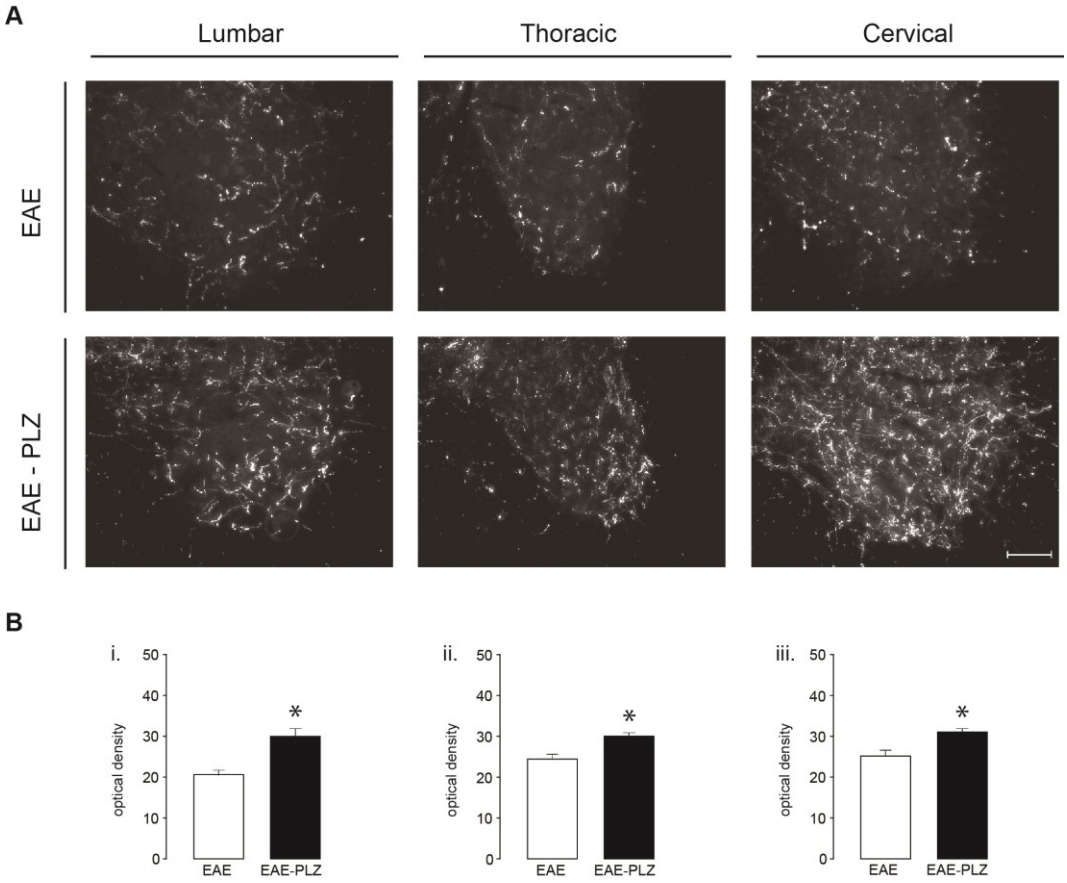

Figure 3.4: PLZ treatment in established EAE leads to significant increases 5-HT immunoreactivity in the ventral horn throughout the spinal cord. (A, B) Cross sections of i) lumbar, ii) thoracic and iii) cervical spinal cords centred on the ventral horn immunostained for 5-HT revealed significant increases in 5-HT immunostaining in PLZ-treated mice with EAE (*P<0.05, t-test). Scale bar = 200 μM and applies throughout. Error bars in (B) panels i, ii, iii, represent SEM.

Figure 3.5

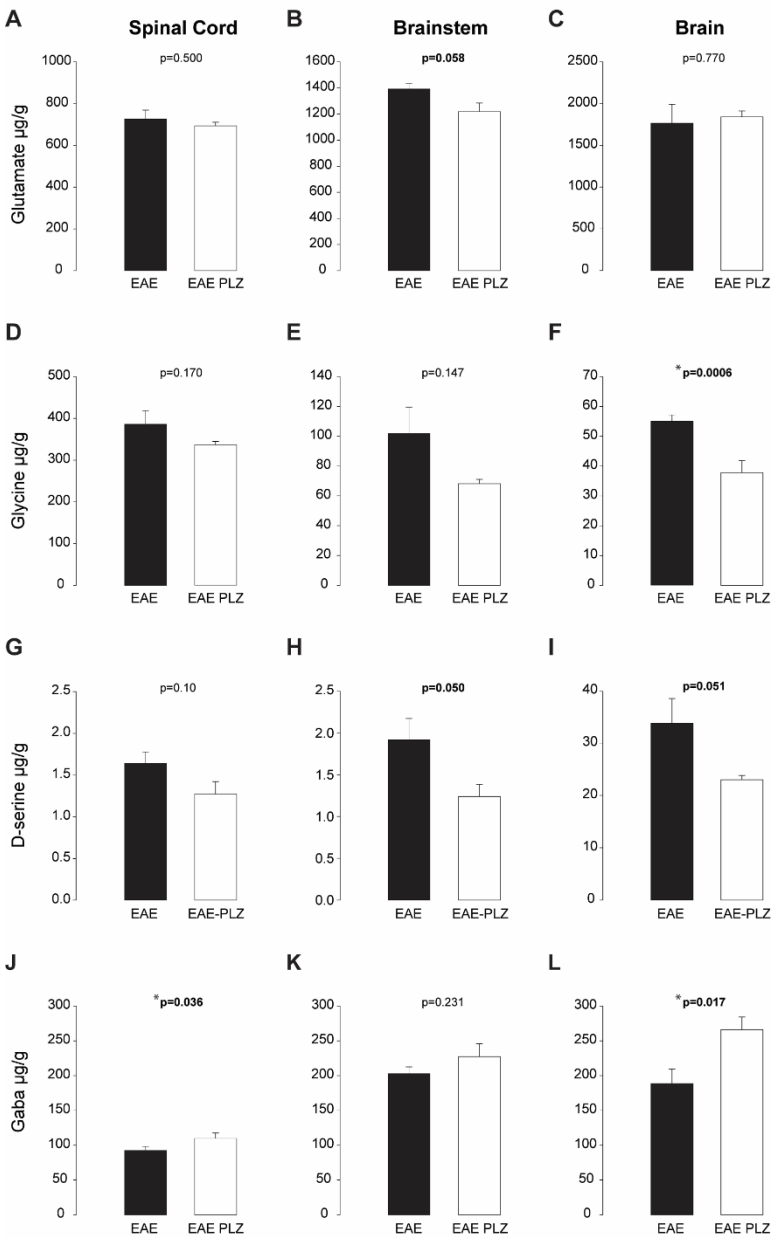

Figure 3.5: PLZ treatment modifies the concentrations of GLU, GLY, D-SER and GABA in established EAE. Concentrations of GLU (A-C), GLY (D-F), D-SER (G-I) and GABA (J-L) in vehicle-treated and PLZ-treated EAE mice from the spinal cord, brain stem brain and brain. (A-C) PLZ did not alter the levels of GLU within the CNS. (D-F) PLZ treatment caused a statistical decrease in the amount of GLY in the brain of EAE mice, but not within the other CNS regions.

(G-I) There was a general decrease in the concentration of D-SER within the CNS. (J-L) PLZ treatment elevated GABA levels in the spinal cord (G and J) and brain (I and L) but not within the brainstem (H and K). Values are mean concentrations in ug/g tissue ± SEM. (* $P<0.05$ t-test).

Figure 3.6

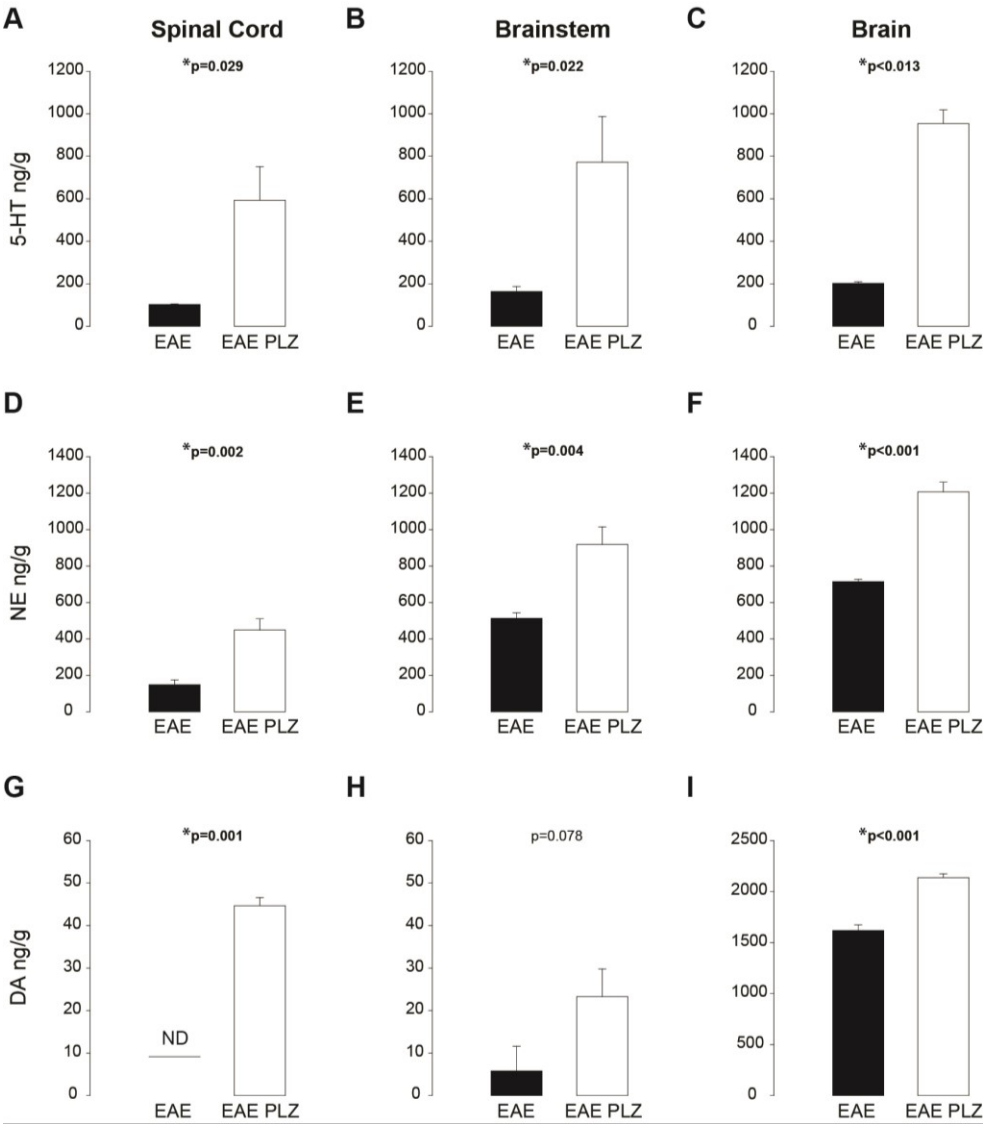

Figure 3.6: PLZ treatment in established EAE increases the concentrations of 5-HT, NE, DA levels in the CNS of mice with EAE. Concentrations of 5-HT (A-C), NE (D-F) and DA (G-I) in vehicle-treated and PLZ-treated EAE mice from the spinal cord, brain stem brain and brain. (A-F) Beginning PLZ treatment at the start of clinical signs significantly elevates the levels of 5-HT and NE within spinal cord, brain and brainstem regions. (G-L) PLZ treatment elevated DA levels in the spinal cord (G) and brain (I) but not within the brainstem (H). Values are mean concentrations in ng/g tissue ± SEM. (* $P<0.05$ t-test).

Figure 3.7

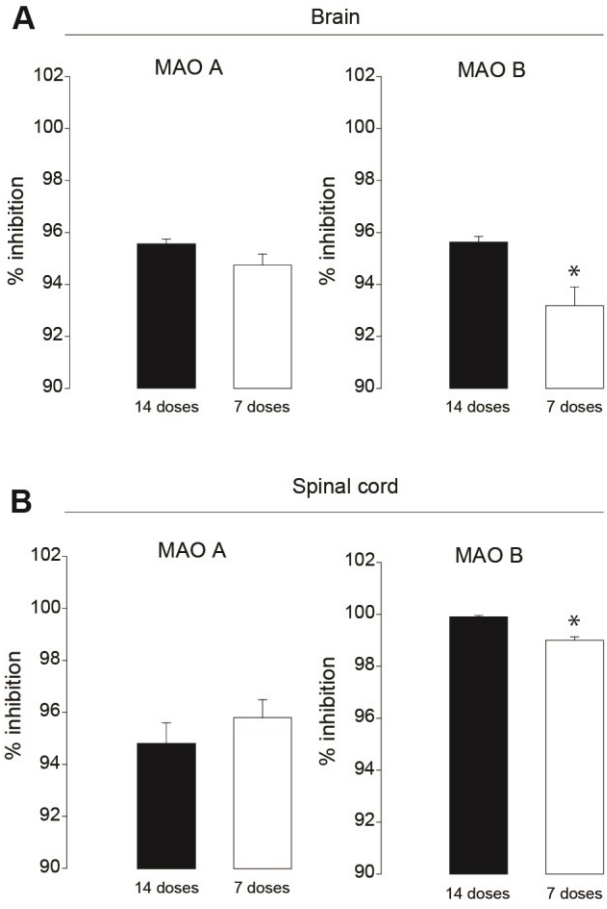

Figure 3.7: PLZ treatment every second day causes less inhibition of MAO B. (A, B) The percent inhibition of MAO-A and MAO-B in the brain (A) and spinal cord (B) from mice treated with PLZ either daily (14 doses) or on alternating days (7 doses). There is no difference in MAO-A inhibition within the brain or spinal cord of EAE mice treated with either 14 or 7 doses of PLZ. In contrast, there was significantly less inhibition of MAO-B in the brain and spinal cord when PLZ is given on alternating days (7 doses) (* $P<0.05$ t-test). Error bars in (A-D) represent SEM.

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Chapter 4

Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE)

4.0 INTRODUCTION

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease of the central nervous system (CNS). The autoimmune attack is thought to generate demyelinated lesions that are the hallmark of early stage MS (Compston and Coles, 2002). These lesions can occur throughout the CNS and can be present in both the white and grey matter. The location and number of lesions can lead to movement impairments that were classically thought to be the critical feature of MS (Brex et al., 2002). However, it is known that increased pain, depression and cognitive problems are also common symptoms that occur over the course of the disease (Hauser and Oksenberg, 2006). Increased pain, in particular central neuropathic pain, is thought to arise from lesions in specific regions of the CNS or a still not completely understood enhancement in CNS pain processing (Cruccu et al., 2009; Olechowski et al., 2010). Consequently, from this lack of understanding, there are currently very few treatment options for pain in patients with MS.

The animal model experimental autoimmune encephalomyelitis (EAE) is a commonly used model to study the pathophysiology of MS (Aharoni, 2013). EAE primarily mimics the autoimmune component of MS; however, our lab and others have shown robust pain hypersensitivity in MOG_{35-55} EAE (Dutra et al., 2013; Olechowski et al., 2010; Olechowski et al., 2009). EAE-associated pain behaviours develop prior to the onset of clinical signs and in the absence of significant demyelination (Olechowski et al., 2009; Rodrigues et al., 2009). This suggests that pathophysiological events that precede motor neuron dysfunction, such as $CD3^+$ T-cell infiltration and glial cell activation in the dorsal horn of the spinal cord, have a role in the abnormal pain response in EAE animals (Olechowski et al., 2009). Indeed, within the dorsal horn, increased activation and number of microglia, astrocytes and T-cells have been observed

prior to the onset of motor symptoms (Olechowski et al., 2009). Methods of modulating these underlying processes may prove beneficial in improving pain hypersensitivity in EAE.

Exercise has been shown to improve many neurological and neurodegenerative diseases (Brown et al., 2013; Pothakos et al., 2009) and a significant amount of clinical research has examined the effects of exercise on MS. Several systematic reviews have indicated that while the benefits of exercise in MS are not yet conclusive, there is an indication that it may improve various MS-related symptoms such as fatigue, depression, anxiety and decreased muscle strength (Asano and Finlayson, 2014; Latimer-Cheung et al., 2013). Current research is being conducted to determine the most effective types of exercise for people living with MS. However, it is unclear whether exercise improves symptoms of pain in the disease. Despite the clinical experience with exercise, only several papers have examined the effects of exercise in EAE (Bernardes et al., 2013; Le Page et al., 1996; Le Page et al., 1994; Patel and White, 2013; Pryor et al., 2014; Rossi et al., 2009) (for review see (Klaren et al., 2014). These papers indicate that forced or voluntary exercise can influence EAE clinical disease, but have yet to measure the effect of exercise on the secondary symptoms associated with EAE.

Therefore, we set out to determine whether voluntary wheel running could improve EAE-associated pain hypersensitivity. The majority of studies examining the effect of voluntary running in EAE allow constant wheel access. To avoid singly housing animals and to provide a more clinically relevant exercise schedule, we allowed only 1 hour/day of access to a running wheel for each mouse in the study. In addition, we tracked the daily distance travelled in order to observe whether it could be used as a measure of disease progression. We show that even limited, 1 hour/day access to a running wheel delays the onset of clinical signs and improves EAE-associated pain hypersensitivity.

4.1 METHODS

4.1.1 EAE Induction and Assessment

All animal studies were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee:Health Sciences for the University of Alberta. EAE was generated in 10 to 12 week old female C57/BL6 mice (Charles River) (n= 60) using myelin oligodendrocytes glycoprotein (MOG) 35-55 obtained from the Peptide Synthesis Facility at the University of Calgary. EAE was induced by subcutaneous injection of 50 μg of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1.5mg/mL. An intraperitoneal injection of 300 ng pertussis toxin (List Biological laboratories, Cedarlane, Canada) was administered on the day of induction and 2 days later. Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1 (disease onset), flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both.

4.1.2 Voluntary Wheel Running

Mice were conventionally housed, 5 animals per cage, with free access to food and water. Mice were removed from their home cage and placed in a similar, standard cage with only bedding and a running wheel (Living World®-Deluxe Exercise Wheel, 5"/12.5cm #61701) for 1 hour per day. One week prior to the induction of EAE, each animal was given 1 hour per day for 3 days to habituate to the running wheel. Starting on day 1 post induction, a group of mice with EAE were allowed one hour of daily access to the wheel (n=30). The distance travelled on the running wheel was recorded using a Schwinn ® 20 Function bike computer (model 04SW654C6PK)

fitted to the running wheel. Animals ran within the same cage and running wheel on consecutive days over the course of the study. Non-running EAE control mice remained in their home cages (n=30).

4.1.3 Mechanical allodynia

Von Frey hair monofilaments calibrated to specific amounts of force were used to assess the sensitivity of EAE mice to mechanical stimuli. This method has been used by several groups to assess pain hypersensitivity in EAE prior to the development of motor impairment (Iannitti et al., 2014; Lu et al., 2012). Mice were placed in clear plexiglass boxes on an elevated wire screen. Increasing amounts of force ranging from 0.04-2.0 g were applied to the plantar surface of each hind paw. Each calibrated filament was applied 5 times/paw and the number of nociceptive behaviours (vigorous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The filament that caused nociceptive behaviours at a frequency of greater than 60% was recorded as the threshold of force required to produce a nociceptive response. Baseline testing was conducted 3 times prior to the induction of EAE. Subsequently, testing was done on day 7 post-induction and on the day a mouse reached the onset of clinical signs (grade 1, flaccid, paralysed tail).

4.1.4 HPLC

Whole spinal cord tissue samples were taken at clinical grade 1 (n=5/30 EAE-run; n=5/30 EAE control). The spinal cord samples were flash frozen in liquid nitrogen and kept at -80 ºC prior to analysis with HPLC. The mobile phase solutions were all made using HPLC grade methanol (MeOH), tetrahydrofuran (THF), and acetonitrile purchased from Fisher Scientific. Standards

and tissue samples were analyzed for amino acids using a minor modification of the procedure of Grant et al. (2006). Tissues were homogenized in 5 volumes of ice-cold H_2O, then diluted 10x with MeOH and left on ice for 10 minutes. The samples were then centrifuged at 12,000xg for 4 minutes and the resulting supernatants were diluted in water to make a 120-fold dilution. Portions of these diluted supernatants were then reacted with *o*-phthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBC) dissolved in borate buffer and analysed using a fluorescence detector set at an excitation wavelength of 344 nm and an emission wavelength of 433 nm. Calibration curves prepared from authentic samples of amino acids were generated for each individual run of samples.

4.1.5 Histology and Immunocytochemistry

Whole spinal cord tissue samples were taken when mice reached clinical grade 1 (flaccid, paralysed tail) (n=5/30 EAE-run; n=5/30 EAE control). In 2 of the 5 EAE-run mice that never reached this clinical grade the spinal cords were taken 28 days after disease induction. Mice were euthanized by an overdose of Euthansol (340mg/ml) and then perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (transcardially). Lumbar spinal cords were removed, post-fixed overnight and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm). To assess inflammation, reactive gliosis, neuronal activity and mitochondrial expression a rat anti-CD3 (1:200, Serotec), rat anti-CD45 (1:200, Serotec), rabbit anti-iNOS (1:500, BD), rabbit anti-Iba-1 (1:500, Wako), and rabbit anti-cFos (1:500, Cell Signalling), rabbit anti-pNR1^{Ser896} (1:500, Cedarlane), mouse anti-Tom20 (1:50, Santa Cruz) and mouse anti-COX

IV (1-250, abcam) were used respectively. These antibodies were visualized using goat anti-rat or goat anti-rabbit Alexa Fluor® 488 (1:200, Invitrogen-Life Technologies, Burlington ON). To visualize cellular nuclei tissue sections were counter stained with mounting media containing fluorescent DAPI (Vector Laboratories)

4.1.6 Quantification of histology and immunocytochemistry

Twenty times magnification images of the spinal cord dorsal horn were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images of whole spinal cord cross sections were captured at 5X magnification using a Leica DMI 6000B microscope. All image analysis was carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. Total cell counts for $CD3^+$ T-cell infiltration, CD45, iNOS, cFOS, and Iba-1 reactivity were made using the Image-based Tool for Counting Nuclei (Centre for Bio-image Informatics, UC Santa Barbara, CA, USA) plugin for NIH ImageJ software. For 5X images this included the entire cross section of the spinal cord and for 20X images only the superficial layers of the dorsal horn (see Figs 3-7 for delineated area of dorsal horn examined). For each stain, a standard total area was measured over the target region within which cells were identified and the cell counting parameters kept constant. To count $pNR1^{Ser896}$ positive cells within the parenchyma of the dorsal horn we used the ImageJ Analyze Particles function with constant cell identification parameters across all images. Tom20 and COX IV were counted manually by an observer blinded to the experimental groups. In all cases DAPI was used to ensure that each stain associated with the nucleus of a cell. All measurements are the average of 2 sections per slide, 1 slide per animal and n=4-5 per group.

4.1.7 Statistical Analysis

Statistical analysis was carried out using the Student's t-test and one-way ANOVA with Tukey *post hoc* tests. For non-parametric data the Mann-Whitney Rank Sum test was used. Significance was set at $P < 0.05$.

4.3 RESULTS

4.2.1 One hour per day of voluntary wheel running delays the onset of EAE

In this experiment, 30/30 of the control mice induced with EAE reached clinical grade 1. Of the 30 mice induced with EAE and allowed 1 hour/day access to a running wheel, 24/30 reached clinical grade 1. In the running group, 1 mouse died shortly after the induction procedure for unexplained reasons (likely a response to the anaesthetics) while 5/30 never reached clinical grade 1. We tracked the distance traveled by the EAE mice given access to the running wheels both prior to and after disease onset (clinical grade 1) (Fig. 1A). Prior to disease onset, EAE mice ran on average 600-800 meters during a 1-hour session. At disease onset these mice displayed a large reduction, only running an average of 290 meters over the hour (Fig 4.1A, arrow). This is despite only showing signs of a paralyzed tail, a hallmark feature of disease onset in EAE. Mice with EAE exhibited almost no voluntary wheel running at the peak of disease (3-6 days post disease onset). However, as the disease progressed, EAE mice partially recovered this voluntary running behaviour. The amount of voluntary wheel running mirrored the EAE disease progression. Since previous reports have indicated that the clinical signs of EAE can be reduced by constant access to voluntary wheels (Rossi et al., 2009), we set out to determine whether a limited exposure of 1 hour of daily running wheel access could attenuate EAE disease progression. We observed that mice given 1 hour per day of access to a running wheel had a

similar peak level of disease and recovered a similar amount as control EAE mice over the course of the experiment (Fig. 4.1C). However, while the overall disease progression appeared similar between the two groups of mice, we observed that mice given access to a running wheel for just 1 hour per day had a significantly delayed onset of these clinical signs by an average of 3-4 days ($p<0.05$, Mann-Whitney Rank Sum test) (Fig. 4.1B).

4.2.2 One hour per day of voluntary wheel running decreases tactile hypersensitivity in EAE

Our lab has previously reported that EAE mice display significant mechanical allodynia at the onset of clinical signs (Olechowski et al., 2009). To determine whether voluntary wheel running could improve this EAE-associated allodynia, Von Frey hair thresholds were assessed in EAE mice pre-symptomatically (day 7 post EAE induction) and at the onset of clinical signs (grade 1) for each mouse. Pain hypersensitivity is difficult to assess during the chronic disease due to hind limb paralysis; therefore, we focused our testing on this early stage of EAE. In order to avoid any acute effects of wheel running on nociceptive sensitivity, Von Frey hair testing was done prior to the mice having access to the running wheel for that day. As previously reported (Iannitti et al., 2014; Lu et al., 2012; Olechowski et al., 2009), control mice with EAE had a significant reduction in their Von Frey hair withdrawal thresholds at clinical grade 1 compared to their baseline values ($p<0.05$, one-way ANOVA, Tukey *post hoc* test) (Fig. 4.2A). In contrast, at the onset of clinical signs in EAE mice with daily access to voluntary running wheels, these thresholds did not show a statistically significant reduction compared to baseline value (EAE no-run control: 42.7 % of baseline threshold, EAE-run: 62.1% of baseline threshold) (Fig. 4.2B).

4.2.3 One hour per day of voluntary wheel running decreases immune cell infiltration in the dorsal horn

To determine whether voluntary wheel running impacted the amount of EAE-associated cellular pathology within the CNS, we first examined demyelination and axonal loss, which are hallmarks of EAE disease. We observed only small and variable amounts of demyelination and axonal loss in both groups of mice at the onset of clinical signs (clinical grade 1). While EAE mice with access to a running wheel tended to have fewer lesions, there were no statistically significant differences in demyelination or axonal loss.

We next used the pan-leukocyte marker CD45 to examine whether the early behavioural improvements in EAE mice given access to the running wheels were due to changes in the gross inflammatory response. Although there was a trend toward a reduction in the number of CD45 positive cells in EAE mice with access to running wheels, the total number of stained CD45 cells did not reach statistical significance compared to no-run, EAE control mice (Fig. 4.3). We next examined the lumbar spinal cord for more specific markers of T-cells (CD3) and macrophages or microglia (iNOS, Iba-1) from tissue taken at disease onset (clinical grade 1). Moreover, we examined whether differences could be observed in whole spinal cord cross sections or more specifically within the superficial lamina of the dorsal horn, a critical region for spinal pain processing (Todd, 2010). Voluntary wheel running reduced the total number of CD3+ cells across whole spinal cord sections compared to control EAE mice ($p=0.054$, t-test) (Fig. 4.4A-B, G). In addition, we observed that voluntary wheel running significantly reduced the number of CD3 positive cells specifically within the dorsal horn of the spinal cord ($p<0.05$, t-test) (Fig.4.4C-D, H).

We next examined iNOS positive macrophages/microglia The level of iNOS expression in wheel running mice was significantly reduced over the entire spinal cord when examined in cross section ($p=0.016$, Mann-Whitney Rank Sum test) (Fig. 4.5A-C). At this early stage of the disease, however, iNOS positive macrophages/microglia were rarely observed within the parenchyma of the superficial dorsal horn in both groups of EAE mice. iNOS positive cells were, however, often observed in the dorsal root and in close proximity to the dorsal root entry zone in control mice with EAE, but this pattern of immunostaining was rarely observed in EAE mice given access to the running wheels (Fig. 4.5 D, E).

We next examined Iba-1 positive cells over the entire cross sectional area of the spinal cord. While there was a trend towards reduced Iba-1 positive cells in EAE mice given access to the running wheels, this did not reach statistical significance (Fig. 4.6A-B, G). However, when we specifically examined Iba-1 cells in the superficial dorsal horn of EAE mice, we found that voluntary wheel running led to a significant reduction in Iba-1 immunostained cells ($p<0.05$, t-test) (Fig. 4.6C-D, H).

4.2.4 One hour per day of voluntary wheel running decreases c-FOS and pNR1 expression in the dorsal horn

The superficial dorsal horn of the spinal cord is critical for the processing and transmission of pain from the periphery to higher brain centres. An increase in the activity of neurons in this region is suggestive of elevated pain processing. To determine whether voluntary wheel running affected neuronal activity in the superficial dorsal horn, we assessed the protein product of the immediate early gene *c-Fos,* a commonly used indicator of neuronal activity (Lin et al., 2014). EAE mice given access to a running wheel had significantly fewer FOS positive cells within the

dorsal horn compared to non-running EAE control mice (EAE control: 33.1±3.8; EAE run: 10.9±2.1; $p<0.05$, t-test) (Fig 4.7A-E).

We next measured the downstream consequences of this increased neuronal activity by examining the levels of the phosphorylated NR1 (pNR1) subunit of the NMDA receptor. We found significantly greater amounts of pNR1 positive cells in the superficial dorsal horn of control EAE mice compared to EAE mice given daily access to the running wheels (EAE control: 61 ±7; EAE run: 27 ±10; $p=0.03$, t-test) (Fig. 4.7 F-J). This suggests that even 1 hour/day of wheel running can dampen the abnormal neuronal activity in the superficial dorsal horn associated with EAE (Olechowski et al., 2010).

4.2.5 One hour per day of voluntary wheel running affects amino acid levels and reduces oxidative stress in the spinal cord

We have previously reported that EAE is associated with decreases in the levels of amino acid and biogenic amine neurotransmitters in the CNS (Benson et al., 2013). To examine whether voluntary wheel running could influence the levels of excitatory or inhibitory amino acids we examined spinal cords at the onset of disease using HPLC. EAE mice given access to a running wheel had elevated levels of the inhibitory neurotransmitter GABA (EAE control: 114.6 ±2.6 μg/g tissue; EAE run: 124.7 ±1.8 μg/g tissue, $p<0.05$, t-test) and although not statistically significant, had a trend towards higher levels of the excitatory neurotransmitter glutamate (EAE control: 1332.2 ± 73.4 μg/g tissue; EAE run: 1532.8 ±65.0 μg/g tissue, $p=0.07$, t-test) (Fig. 4.8A-B).

In addition, using HPLC we measured the levels of glutathione (GSH) and glutathione disulfide (GSSG). GSH is a critical antioxidant and when oxidized becomes GSSG. Oxidative

stress has been shown to increase GSSG levels and is associated with a decrease in the ratio of GSH to GSSG (GSH/GSSG) (Lew et al., 1985). Levels of GSH were significantly higher in EAE mice that ran daily compared to controls (EAE control: 229.0 ±9.1 μg/g tissue; EAE-run: 260.8 ±9.1 μg/g tissue, $p<0.05$, t-test) (Fig. 4.8C). Voluntary wheel running was associated with a significant decrease in the levels of GSSG compared to control EAE mice (EAE control: 11.7 ±0.6 μg/g tissue; EAE run: 7.5 ±1.4 μg/g tissue, $p<0.05$, t-test) (Fig. 4.8D). The ratio of GSH/GSSG was significantly greater in EAE mice given access to the running wheels compared to EAE controls ($p<0.05$, Mann Whitney Rank sum) (Fig. 4.8E). This indicates that voluntary wheel running in mice with EAE can lead to a reduction in oxidative stress at the spinal level.

4.2.6 One hour per day of voluntary wheel running decreases the expression of the mitochondrial proteins COX IV and Tom20

Disrupted ATP production and changes in reactive oxygen species (ROS) due to mitochondrial dysfunction have been suggested to play a role in the neurodegeneration associated with MS. In addition, increased levels and activity of mitochondrial proteins have previously been observed in MS lesions (Witte et al., 2009). Given the effects of voluntary wheel running on markers of oxidative stress, we next assessed the expression of the mitochondrial proteins COX IV and Tom20 in the dorsal horn of the spinal cord. The number of COX IV positive cells in the dorsal horn of the spinal cord was significantly lower in the EAE mice with access to a running wheel compared to EAE controls (EAE control: 72.7±24.9; EAE run: 204.8 ±6.8; $p<0.05$, t-test) (Fig. 4.9 A-E). Similarly we observed significantly fewer Tom20 positive cells within the superficial lamina of the spinal cord of EAE mice given access to the running wheels (EAE control: 169.5±32.6; EAE run: 29.3 ±15.3; $p<0.05$, t-test) (Fig. 4.9 F-J). These observations demonstrate

that even 1 hour a day of voluntary wheel running is able to influence the expression of mitochondrial proteins within the dorsal spinal cord of EAE mice.

4.3 DISCUSSION

People living with MS are interested in interventions that can improve their quality of life in addition to disease-modifying therapies. Exercise is becoming an important component in the management of MS. There has been positive evidence for the benefits of exercise in MS but the effects of exercise on MS-related pain is understudied. Furthermore, the mechanisms underlying the benefits of exercise in MS-related secondary symptoms require more research. Our current study indicates that EAE mice given 1 hour/day of access to a voluntary wheel have a delay in the onset of clinical signs and improved pain hypersensitivity early in the disease. These behavioural improvements were associated with reduced immune infiltration and lower oxidative stress. It is likely that exercise is exerting various effects in EAE and that the complete mechanisms behind these results are complex.

In EAE the effects of exercise on the immune response are not completely clear. A recent study reported that swimming reduced the expression of TNF-α, IL-β, IL-6 and Il-10 within the brains of mice with EAE (Bernardes et al., 2013). Despite these changes there was no effect on immune cell infiltration into the CNS. In addition, EAE mice that received 10 days of forced treadmill running had lower whole brain TNF-α levels; however immune infiltrates were not measured in this report (Patel and White, 2013). Rossi and colleagues also reported that voluntary wheel running did not influence immune cell infiltrates in EAE and attributed the beneficial effects to preventing dendritic spine loss (Rossi et al., 2009). In our experiment we observed that 1 hour/day of voluntary wheel running reduced the number of $CD3^+$ T-cells in the

spinal cord. This limited access to running wheels also significantly reduced the signs of microglia/macrophage reactivity within the dorsal horn of the spinal cord, an important site for pain processing in the CNS. Our measurements were taken at the onset of clinical signs (tail paralysis) that were delayed approximately 3-5 days in mice given access to the running wheels. While it is clear that voluntary running does not prevent the transmigration of immune cells into the CNS, it is possible that it slows their entry. However, over time, T-cells can mediate sufficient damage to cause paralysis and motor impairments even if the overall load is reduced. Furthermore, we observed that 1 hour/day of wheel running did not influence the disease course once established compared to controls. This is probably a consequence of the limited amount the animals in our experiment were exposed to a running wheel. Two papers have tested voluntary wheel exercise in EAE and have shown a moderate effect on disease progression (Pryor et al., 2014; Rossi et al., 2009). In these cases the animals had access 24 hours/day 7 days/week, which is a good proof of concept but perhaps not entirely clinically relevant. Further work is needed to determine the optimal amount of exercise in EAE. Of note is that control mice remained in their home cage while experimental mice were placed with running wheels for 1 hour per day. While enriched environments can have a beneficial effect on EAE, these improvements were linked to increased neurogenesis and not to any of the measures improved by voluntary wheel running (Magalon et al., 2007). Furthermore, it is unlikely that the delay in the onset of clinical signs was due to increased stress in response to the running wheels. Voluntary wheel running has been observed in wild mice and is doubtful that wild animals would participate in something stressful (Meijer and Robbers, 2014). Despite this we cannot completely exclude the possibility that a novel environment plays a role in some of the improvements we observed.

In healthy subjects exercise can have various effects on the immune environment. Sustained intense exercise can induce a transient immune suppression by reducing lymphocyte number and reducing the phagocytic function of innate immune cells (Kakanis et al., 2010; Nieman and Pedersen, 1999). However, moderate acute exercise may enhance the immune response through increases in inflammatory cytokine production within muscle (Peake et al., 2005). While the type of acute exercise appears to have differing effects on the immune system, it is established that chronic exercise is anti-inflammatory (Kohut et al., 2006). Classically the anti-inflammatory effects of exercise have been thought to be the result of a shift in the cytokine profile of immune cells (Ostrowski et al., 1999). However, this is a complex phenomenon and likely involves actions on immune cell signalling, adipose tissue composition and innate immune activation (Lancaster and Febbraio, 2014). Our data demonstrating reduced levels of CD3+ positive T-cells infiltrating into the spinal parenchyma and more specifically the decreased levels of iNOS+ macrophages are indicative of an overall anti-inflammatory effect of voluntary wheel running in mice with EAE.

The changes in pain behaviours associated with EAE are thought to develop as a result of the immune and glial responses that can promote sensitization of pain pathways in the CNS. This occurs through the influx of $CD3^+$ T-cells into the dorsal horn of the spinal cord. In particular, the accumulation of Th1 and Th17 subsets leads to a release of proinflammatory cytokines that are able to directly sensitize pain afferents (Kawasaki et al., 2008). This proinflammatory environment can also lead to the activation of resident glial cells, which has been linked to the initiation and maintenance of neuropathic pain (Scholz and Woolf, 2007). One hour per day of voluntary wheel running led to a significant reduction in pain behaviours at the onset of clinical signs. This improvement was paralleled by reduced numbers of $CD3^+$ T-cells in the dorsal horn

and a decreased amount of Iba-1 reactivity within this region. There is some evidence that exercise can modulate the activity of glial cells (Bernardi et al., 2013; Cobianchi et al., 2010). However, there is minimal research investigating the connections between exercise, glia cells and neuropathic pain. Even less has been done to examine this relationship in neurodegenerative diseases such as MS or the animal model EAE. To our knowledge, this is the first report that pain hypersensitivity can be reduced by wheel running in EAE.

GABA and glutamate are both critical for normal CNS function. They have opposing effects on signalling within the CNS; glutamate acts as the principal excitatory neurotransmitter and GABA is principally for inhibitory neurotransmission. Their role in the progression of EAE has been investigated extensively. The metabolism of GABA and glutamate and neurotransmission by these key neurotransmitters are both impaired in EAE (De Chiara et al., 2013; Paul et al., 2014). We have previously shown that the levels of both GABA and glutamate decrease in the spinal cord with EAE progression (Musgrave et al., 2011b). This likely occurs from increasing amounts of immune-mediated neuronal cell death (Anderson et al., 2008). In the current experiment, EAE mice that had access to voluntary wheel running had higher levels of GABA and a trend towards increased glutamate. This suggests that even this limited amount of exercise can maintain a healthier spinal cord in which the balance of excitatory and inhibitory transmission is not perturbed. This is reflected by the reduced numbers of FOS-positive cells in the superficial dorsal horn of EAE mice given access to the running wheels. In addition, we also observed significantly fewer cells positive for the phosphorylated NR1 subunit (pNR1) of the NMDA receptor in the superficial dorsal horn of these mice. pNR1 has been previously been shown to be critical for the development of central sensitization and pain hypersensitivity. We have previously demonstrated that EAE is associated with a large increase in basal FOS

expression in the superficial dorsal horn and increased levels of pNR1 (Olechowski et al. 2010). The diminished numbers of FOS-positive cells in EAE mice allowed to run for 1 hour/day suggest that early in the disease, ongoing cellular activity driving FOS expression in the diseased spinal cord can be normalised by exercise. The decreased FOS expression and lower levels of pNR1 in wheel running EAE mice also correlates with their attenuated pain hypersensitivity.

In addition to the changes in neurotransmitter levels, EAE and MS are associated with increased oxidative stress. In EAE, oxidative stress is elevated primarily due to the release of ROS from activated macrophages and glial cells (Lassmann and van Horssen, 2011). This oxidative imbalance promotes further inflammation, demyelination and neuronal loss (di Penta et al., 2013). To measure the effects of voluntary wheel running on CNS oxidative activity we measured the ratio of GSH/GSSG. GSH is a critical cellular antioxidant for the protection against normal oxidative stress (Pompella et al., 2003). The ratio of reduced (GSH) to oxidized (GSSG) glutathione is a measure of the overall oxidative activity within a system (Schafer and Buettner, 2001). We observed that voluntary wheel running caused an increase in the ratio of GSH/GSSG, which suggests that there is more glutathione in the reduced form and therefore less ongoing oxidative stress. This is another indicator that voluntary wheel running in EAE is able to promote a healthier CNS environment. Moreover, reducing oxidation by using an ROS scavenger has also been shown to improve neuropathic pain behaviour in a rat model of peripheral neuropathy (Kim et al., 2004).

ROS within the cell is tightly controlled by several dedicated pathways. Major regulators of ROS production are the mitochondria themselves. Mitochondria both produce and sequester ROS. In MS this balance can become disrupted due to the ongoing inflammatory processes causing significant mitochondrial dysfunction. In particular, the large amounts of ROS released

by activated microglia and macrophages can increase mitochondrial ROS production and dysfunction (Nikic et al., 2011). Mitochondrial dysfunction as a result of ROS mediated damage and energy loss can contribute to neurodegeneration over the course of the disease. In addition to these long-term consequences, mitochondria are known to have a role in normal and abnormal pain transmission. Unmyelinated fibers have higher energy demands and more mitochondria per area compared to myelinated neurons (Wang et al., 2008a). In addition, mitochondrial Ca^{2+} uptake and ROS production is required for the development of NMDA receptor-mediated hyperalgesia through increased amounts of the protein kinases: PKA, PKC and pERK (Kim et al., 2011). Disruption of these normal mitochondrial processes could lead to maladaptive pain situations. For example, mitochondrial dysfunction has been associated with chemotherapy, diabetic, and HIV induced painful neuropathies (Flatters, 2015). In the present study we observed that in addition to a reduction in activated microglia and oxidative stress, voluntary wheel running in EAE also reduced mitochondrial protein expression in the dorsal spinal cord compared to non-running control mice with EAE. EAE and MS have been associated with an elevated number of mitochondria. This has been suggested to be a consequence of the increased energy demands of damaged or demyelinated axons (Kiryu-Seo et al., 2010; Mahad et al., 2009; Witte et al., 2009). Although this may be a compensatory mechanism, in the context of a sensory nerve, these mitochondrial changes may increase pain hypersensitivity.

In summary, we have demonstrated that when mice with EAE are given daily access to a voluntary running wheel for even a brief period (1 hour/day), it can delay the onset of disease and decrease pain hypersensitivity early in the course of disease. These improvements are associated with reduced inflammatory markers in the spinal cord and less FOS and pNR1 positive cells within the dorsal horn. In addition, voluntary wheel running resulted in less

oxidative stress within the spinal cord. Importantly, these results were achieved with only limited access (1 hour per day) to a running wheel. Exercise can have an impact on a number of diseases. Here we show that 1 hour/day of physical activity can improve pain and delay the onset of clinical signs in a mouse model of MS. This sets the stage for future studies to assess the role of exercise on pain hypersensitivity in the chronic stages of EAE.

4.4 FIGURES

Figure 4.1

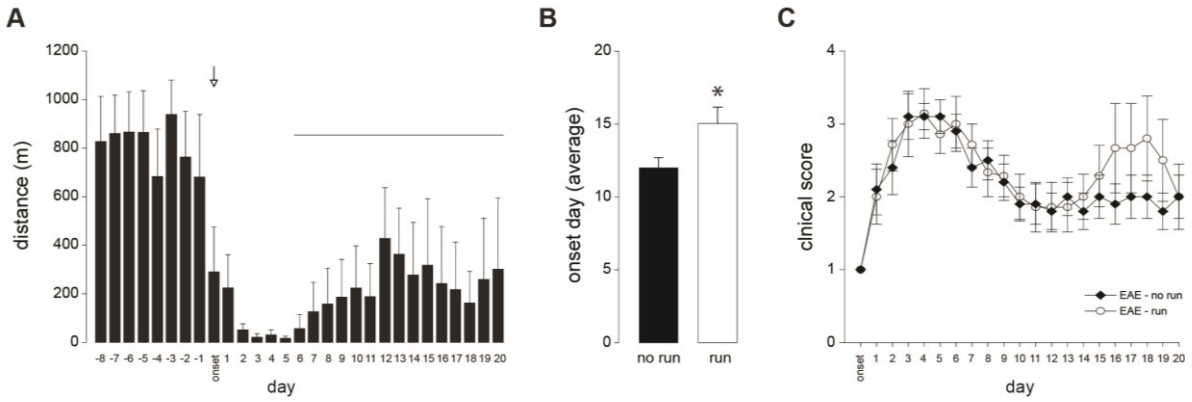

Figure 4.1: Voluntary wheel running and EAE. (A) Average distance run by mice during a 1-hour exposure to a running wheel in the week up to the 'onset' of clinical signs (arrow) and 21 days after (solid line). At the disease onset mice ran an average of 290 meters within the hour. EAE mice displayed a slight recovery in wheel running behaviour over the course of the disease. (B) The average number of days to the onset of clinical signs after disease induction. Mice without exposure to running wheels begin to show clinical signs on average, 11 days after induction. Voluntary wheel running significantly delays the onset by approximately 3 days (*P<0.05, Mann-Whitney rank sum test). (C) The disease progression of EAE mice with access to a running wheel and control EAE mice. After the onset of clinical signs (clinical grade 1) the disease course of EAE is similar between mice with and without access to a running wheel. EAE no-wheel n=15; EAE wheel n=15. Error bars in (A-C) represent SEM.

Figure 4.2

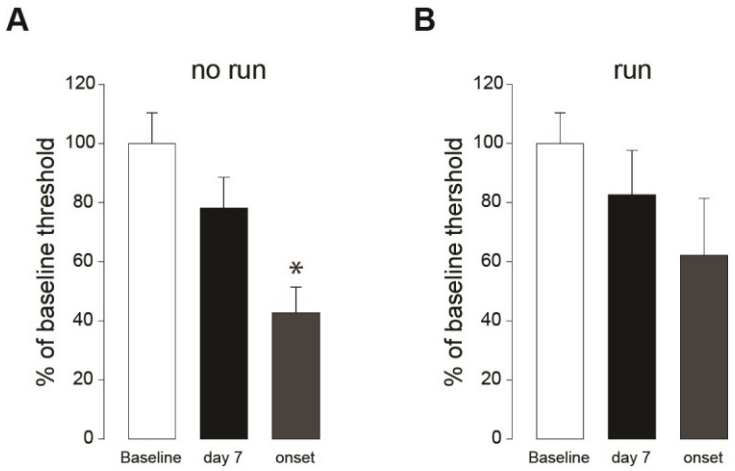

Figure 4.2: Voluntary wheel running and pain sensitivity. (A) Withdrawal thresholds to Von Frey hair mechanical stimulation are significantly decreased (i.e. tactile allodynia) in control EAE mice not given access to running wheels when they reach clinical grade 1 (”onset”) compared to their baseline values (*P<0.05, one-way ANOVA on ranks). (B) There is not a significant difference in the withdrawal thresholds to von Frey hair mechanical stimulation in the EAE mice that have access to running wheels (1 hour/day). (Error bars in (A-B) represent SEM, EAE control n=15; EAE run n=15).

Figure 4.3

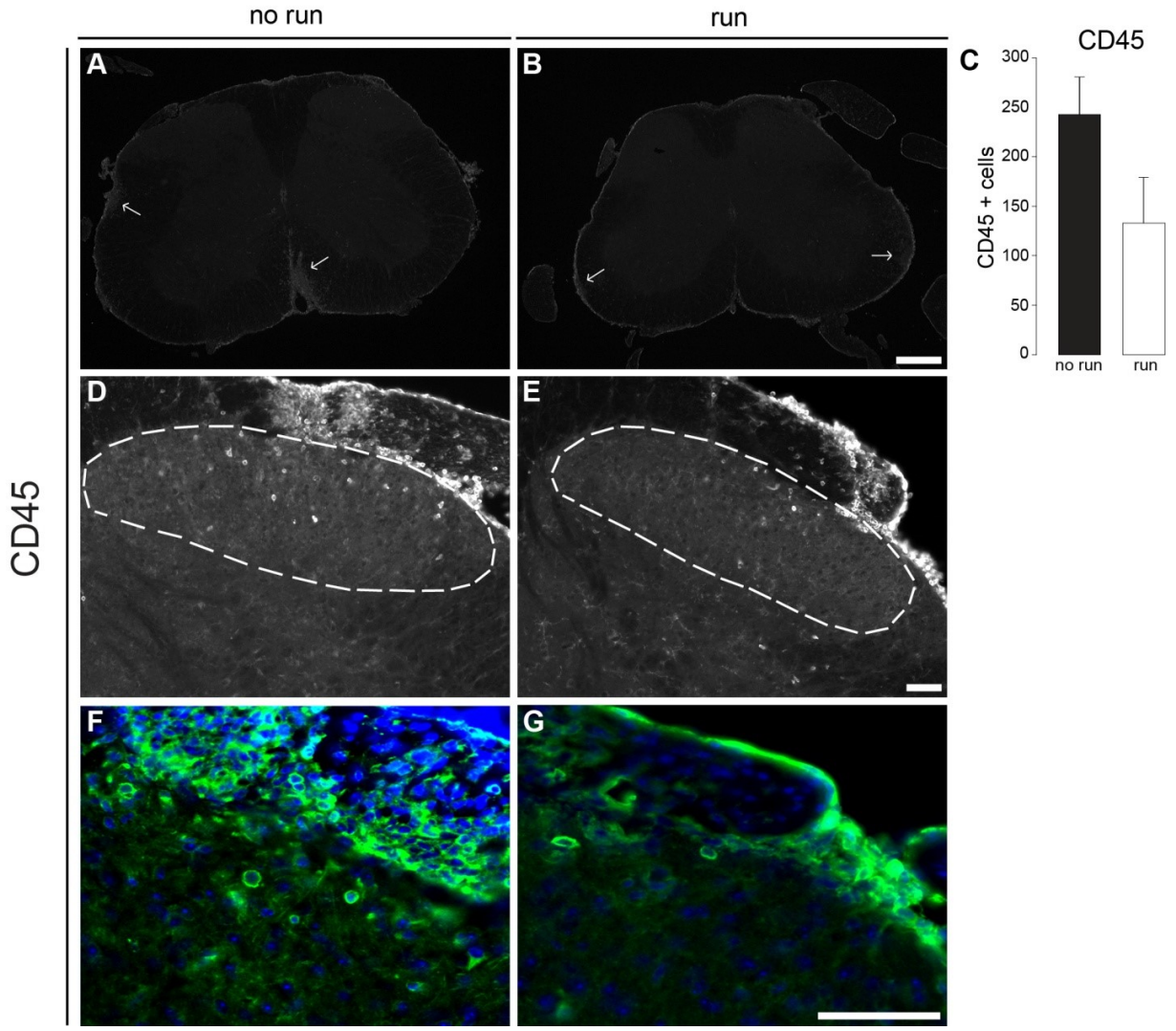

Figure 4.3: Voluntary wheel running and CD45 staining. (A-B) Representative 5X lumbar spinal cord images stained with anti-CD45 from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a running wheel (B). Arrows in (A) and (B) indicate regions with significant inflammation. (C) Voluntary wheel running has a slight but not statistically significant effect on the overall number of CD45 staining within lumbar spinal cord sections. (D-E) Representative 20X images of the dorsal root, dorsal root entry zone and superficial dorsal horn. CD45 numbers were generally lower in mice with EAE given access to

the running wheels (E) compared to spinal cords from EAE controls (D). (F-G) Representative spinal cord images indicating that CD45 staining is associated with dapi positive nuclei in both EAE controls (F) and EAE mice with access to a running wheel (G). (EAE control n=4; EAE run n=5). Scale bar in (B)=250μm and applies (A-B). Scale bar in (E)=50μm and applies to (D-E). Scale bar in (G)=50μm and applies to (F-G). Error bars in (C) represent SEM.

Figure 4.4

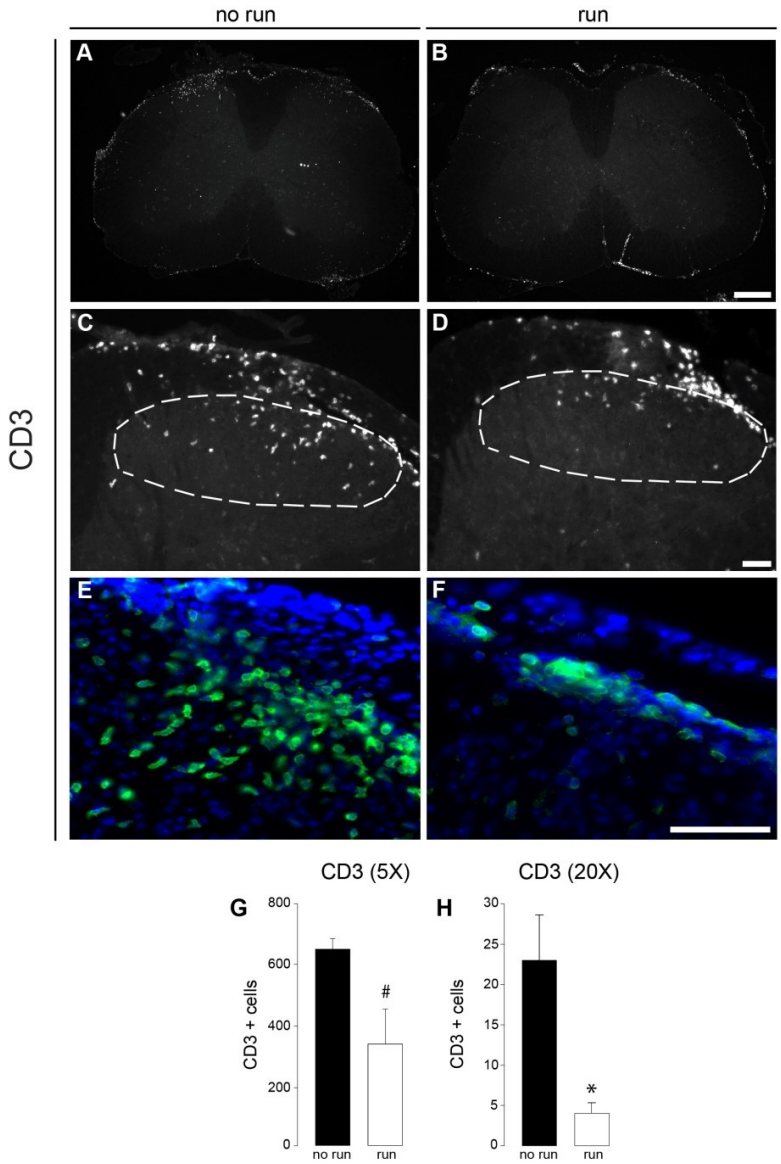

Figure 4.4: Voluntary wheel running reduces the number of T-cells in the spinal cord at the onset of clinical signs. (A-B) Representative 5X lumbar spinal cord images from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a running wheel (B) stained for CD3. (H) Voluntary wheel running reduced the total number of T-cells infiltrating in the spinal cord at clinical grade 1, ($^{\#}$P=0.054, t-test). (C-D) Representative 20X images of the spinal dorsal horn from a control mouse with EAE (C) and a mouse with EAE given 1hour/day access to a

running wheel (D). (G) The number of T-cells in the dorsal horn is significantly less in mice that have access to the running wheels (*P<0.05, t-test). (E-F) Representative spinal cord images indicating that CD3 staining is associated with dapi positive nuclei in both EAE controls (E) and EAE mice with access to a running wheel (F). (EAE control n=4; EAE run n=5). Scale bar in (B)=250μm and applies (A-B). Scale bar in (D)=50μm and applies to (C-D). Scale bar in (F)=50μm and applies to (E-F). Error bars in (G-H) represent SEM.

Figure 4.5

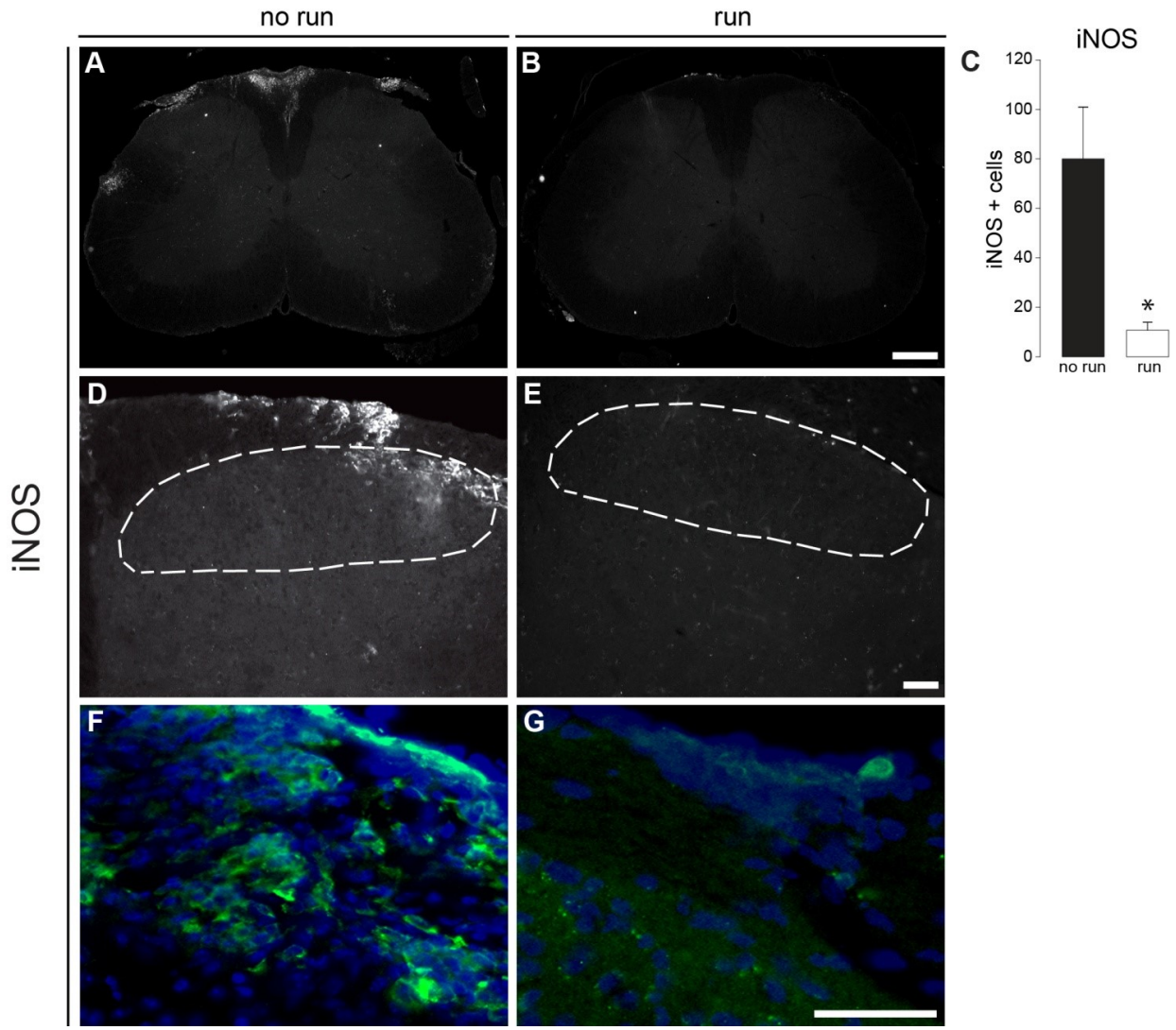

Figure 4.5: Voluntary wheel running reduces iNOS levels in the spinal cord at the onset of clinical signs. (A-B) Representative 5X images of iNOS stained lumbar spinal cord sections from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a running wheel (B). (C) There is a significant reduction in the overall number of iNOS positive cells, (*$P<0.05$, t-test). (D-E) Representative 20X images of iNOS staining in the spinal dorsal horn. Very few iNOS positive cells were detected in dorsal horn of either group although the levels of iNOS are noticeably higher around the dorsal root entry zone in control EAE mice (D). (F-G)

Representative spinal cord images indicating that iNOS staining is associated with dapi positive nuclei in both EAE controls (F) and EAE mice with access to a running wheel (G). (EAE control n=4; EAE run n=5). Scale bar in (B)=250μm and applies to (A-B). Scale bar in (E)=50μm and applies to(D-E). Scale bar in (F)=50μm and applies to (F-G). Error bars in (C) represent SEM.

Figure 4.6

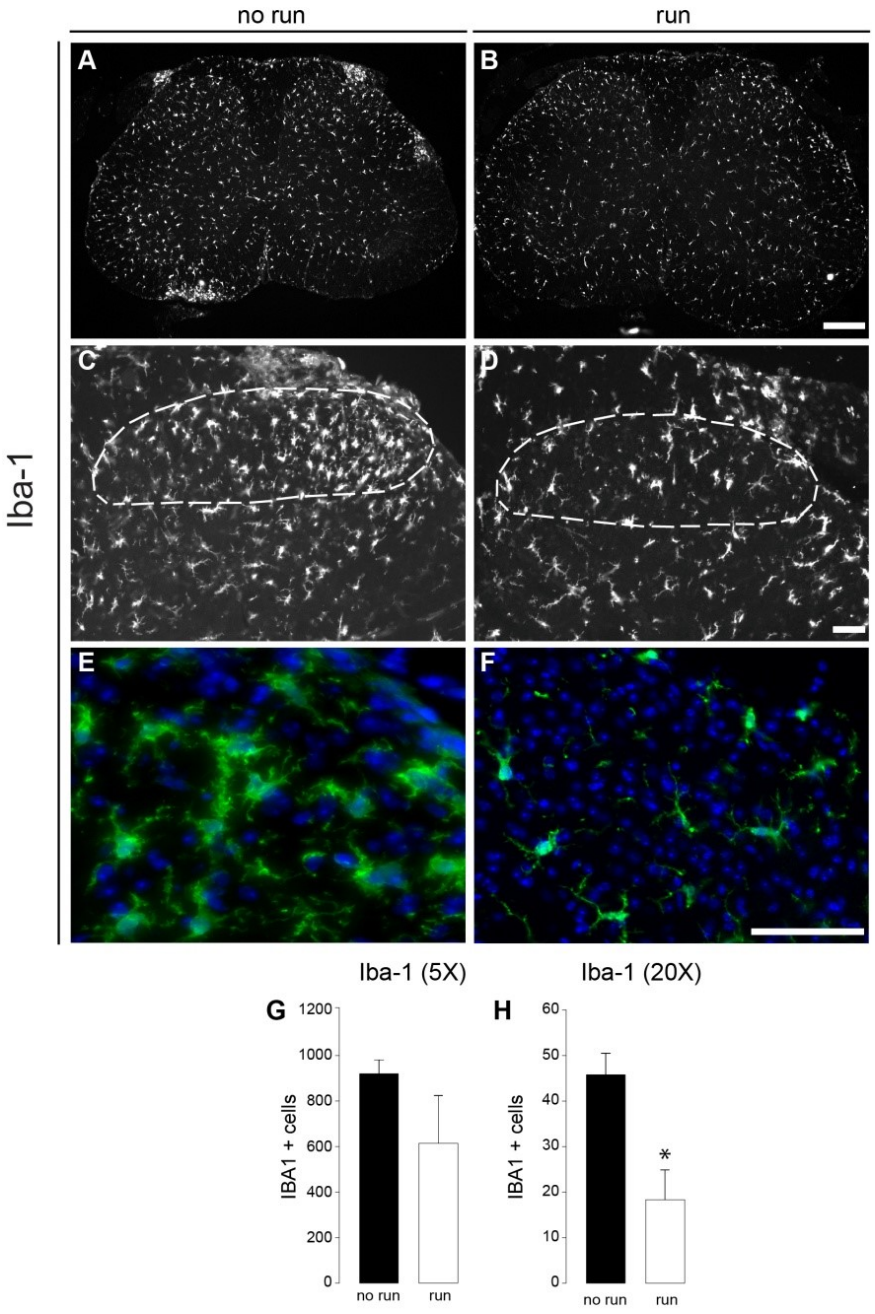

Figure 4.6: Voluntary wheel running reduces Iba-1 levels in the spinal cord at the onset of clinical signs.

(A-B) Representative 5X images of Iba-1 stained lumbar spinal cord sections from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a running wheel (B). (G)

There is a trend but not a statistically significant reduction in the overall number of Iba-1 stained cells. (C-D). Representative 20X images of Iba-1 staining in the spinal cord dorsal horn from a control mouse with EAE (C) and a mouse with EAE given 1 hour/day access to a running wheel (D). (H) The number of Iba-1 positive cells in the spinal dorsal horn is significantly lower in EAE mice given access to the running wheels compared to control mice with EAE (*$P<0.05$, t-test). (E-F) Representative spinal cord images indicating that Iba-1 staining is associated with dapi positive nuclei in both EAE controls (E) and EAE mice with access to a running wheel (F). (EAE control n=4; EAE run n=5). Scale bar in (B)=250μm and applies (A-B). Scale bar in (D)=50μm and applies to(C-D). Scale bar in (F)=50μm and applies to (E-F). Error bars in (G-H) represent SEM.

Figure 4.7

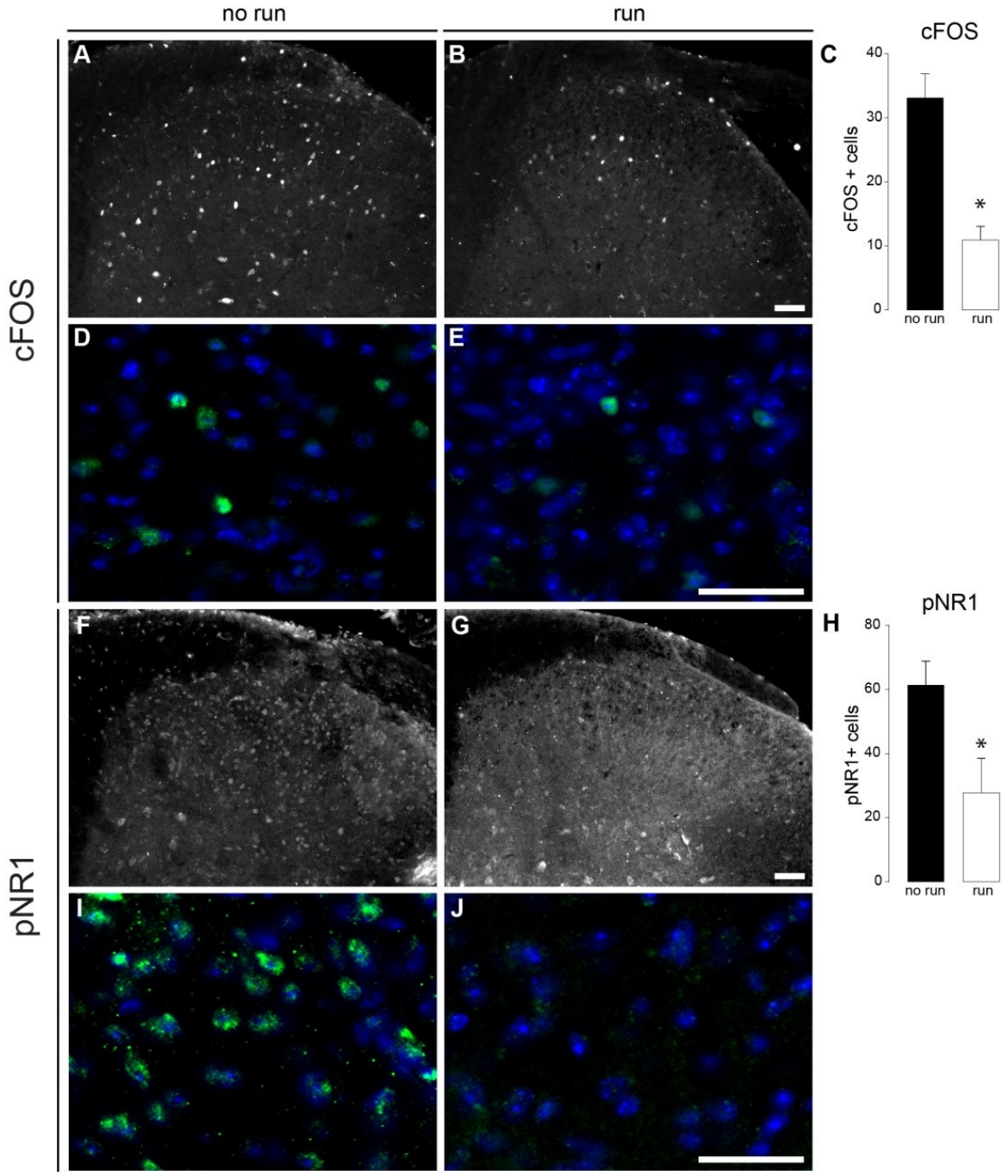

Figure 4.7: Voluntary wheel running reduces FOS expression and pNR1 levels in the dorsal horn of the spinal cord. (A-B) Representative 20X images of FOS staining in the spinal dorsal horn from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a

running wheel (B). (C) There is a large number of FOS-positive cells within the superficial lamina of the lumbar spinal cord in control EAE mice. EAE mice with access to a running wheel have significantly fewer FOS-positive cells compared to controls (*P<0.05, t-test). (F-G) Representative 20X images of pNR1 staining in the spinal dorsal horn from a control mouse with EAE (F) and a mouse with EAE given 1 hour/day access to a running wheel (G). (H) The number of pNR1 positive cells within the dorsal horn of the spinal cord is significantly less in EAE mice with access to a running wheel compared to control EAE mice (*P<0.05, t-test). (D-E, I-J) Representative spinal cord images indicating that cFOS (D-E) and pNR1 (I-J) staining is associated with dapi positive nuclei in both EAE controls (D,I) and EAE mice with access to a running wheel (E,J). (EAE control n=4; EAE run n=5). Scale bar in (B)=250μm and applies (A, B). Scale bar in (E)=50μm and applies to (D-E). Scale bar in (G)=50μm and applies to (G-F). Scale bar in (J)=50μm and applies to (I-J). Error bars in (C, H) represent SEM.

Figure 4.8

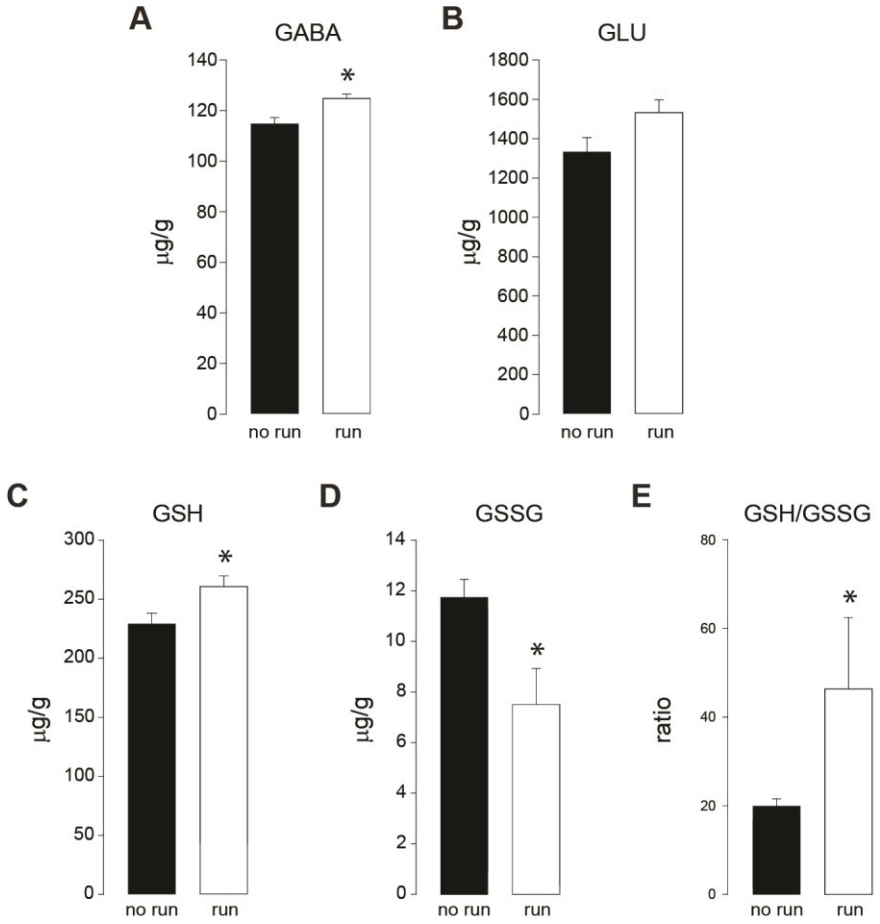

Figure 4.8: Voluntary wheel running increases the concentrations of GABA, glutamate and the GSH/GSSG ratio the spinal cord of mice with EAE. Concentrations of GABA (A), glutamate (B), GSH (C) and GSSG (D) are expressed as µg per gram of tissue. At clinical grade 1 (disease onset), EAE mice with one hour of daily access to a running wheel have significantly higher levels of GABA, and GSH within the spinal cord compared to control EAE mice (*P<0.05, t-test). (D) Voluntary wheel running leads to a significant reduction in the levels of GSSG in EAE mice (*P<0.05, t-test). (E) The ratio of GSH/GSSG was significantly higher in EAE mice with 1

hour daily access to a running wheel compared to EAE controls (*P<0.05, t-test). Error bars in (A-E) represent SEM.

Figure 4.9

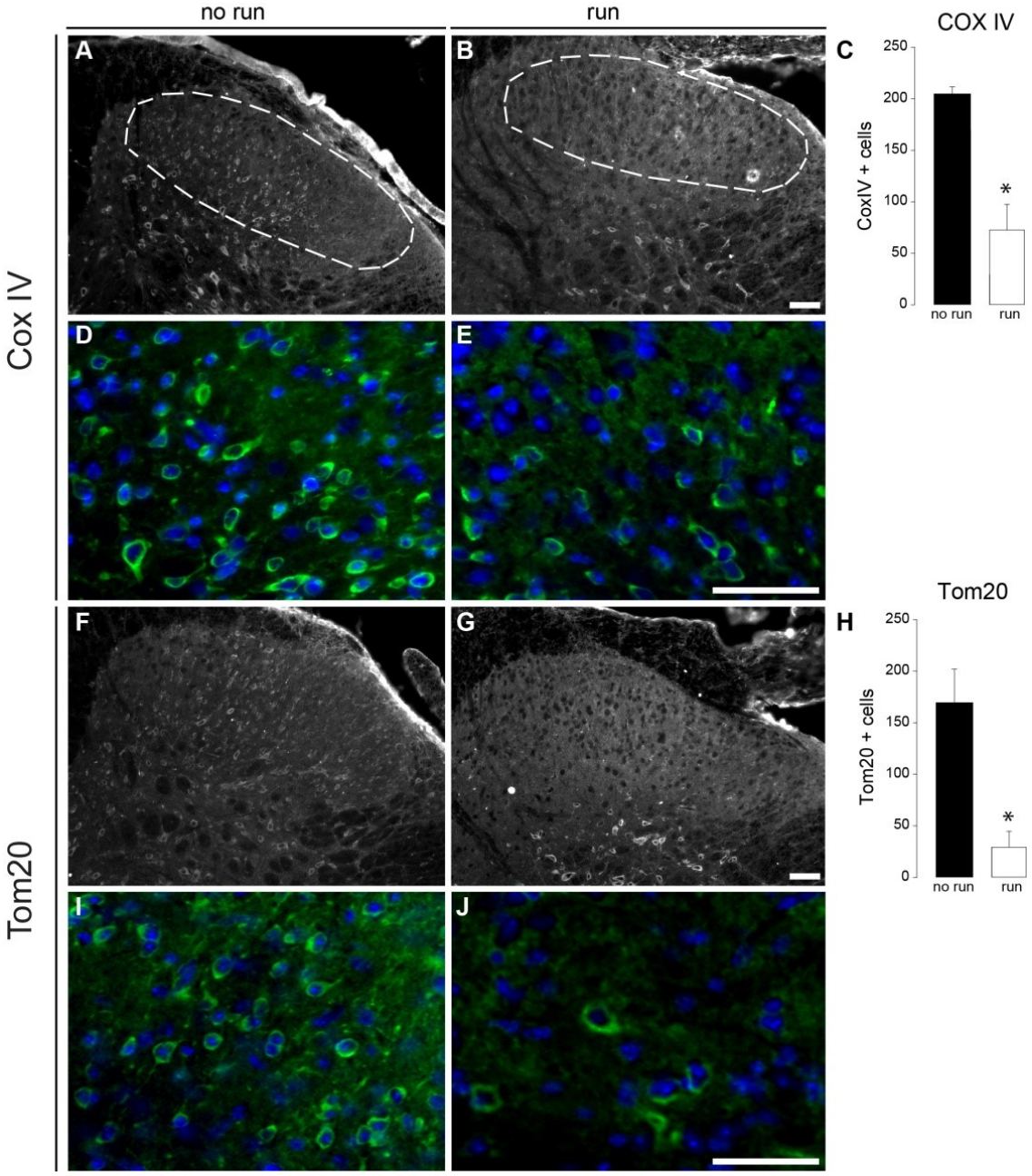

Figure 4.9: Voluntary wheel running reduces the expression of the mitochondrial proteins COX IV and Tom20 in the dorsal horn of the spinal cord. (A-B) Representative 20X images of COX IV staining in the spinal dorsal horn from a control mouse with EAE (A) and a mouse with EAE given 1 hour/day access to a running wheel (B). (C) There is a significant number of COX IV

positive cells within the superficial lamina of the lumbar spinal cord in control EAE mice. EAE mice with access to a running wheel have significantly fewer COX IV positive cells compared to controls (*P<0.05, t-test). (F-G) Representative 20X images of Tom20 staining in the spinal dorsal horn from a control mouse with EAE (F) and a mouse with EAE given 1 hour/day access to a running wheel (G). (H) The number of Tom20 positive cells within the dorsal horn of the spinal cord is significantly less in EAE mice with access to a running wheel compared to control EAE mice (*P<0.05, t-test). (D-E, I-J) Representative spinal cord images indicating that COX IV (D-E) and Tom20 (I-J) staining is associated with dapi positive nuclei in both EAE controls (D,I) and EAE mice with access to a running wheel (E,J). (EAE control n=4; EAE run n=5). Scale bar on (B)=250μm and applies (A-B). Scale bar in (E)=50μm and applies to (D-E). Scale bar in (G)=50μm and applies to(G-F). Scale bar in (J)=50μm and applies to (I-J). Error bars in (C-H) represent SEM.

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Chapter 5

Altered mitochondrial protein expression within dorsal spinal cord during early EAE

5.0 INTRODUCTION

Multiple sclerosis MS is an autoimmune neurodegenerative disease of the central nervous system. MS is a lifelong disease with the first symptoms arising between the ages of 20-40 years (Compston and Coles, 2002). In recent years there has been a substantial increase in the number of effective treatments aimed at reducing the inflammatory infiltration of the CNS. However, despite the success of these drugs at preventing relapses, they fail to stop disease progression and the secondary symptoms of MS (Haghikia et al., 2013). In particular, these drugs have not diminished the prevalence of pain in people with MS (Foley et al., 2013). Pain in MS is associated with a decreased quality of life and is often paralleled by depression and cognitive impairment (Kalia and O'Connor, 2005). Pain hypersensitivity can also be detected in various animal models of MS. Our lab and others have described increased thermal and mechanical pain in MOG_{35-55} experimental autoimmune encephalomyelitis (EAE) (Dutra et al., 2013; Olechowski et al., 2010; Olechowski et al., 2009). These pain behaviours begin in early EAE, prior to the onset of clinical motor impairment (Olechowski et al., 2009). Recent work in our lab also found that daily voluntary wheel running is able to reduce the symptoms of pain in EAE (see chapter 4). This improvement was associated with less oxidative stress and reduced mitochondrial protein expression in the dorsal spinal cord, an area of the spinal cord critical for sensory processing (see chapter 4).

The role of mitochondria in sensory processing and pain has only started to become clear over the last decade. Many studies have revealed that mitochondrial function is important for normal and abnormal pain signalling (for review see (Flatters, 2015). In particular, mitochondrial Ca^{2+} buffering is critical for normal sensory function and pain (Kim et al., 2011; Medvedeva et al., 2008; Shutov et al., 2013). Kim et al., 2011 showed that NMDA receptor-evoked

hypersensitivity could be blocked using an inhibitor of the mitochondrial calcium uniporter (Kim et al., 2011). In addition, the authors provided evidence that spinal long term potentiation (LTP) was dependent on Ca^{2+} being sequestered by mitochondria (Kim et al., 2011). Furthermore, in models of neuropathic pain, significant increases in mitochondrial number have been observed in laminae I-V of the spinal cord on the ipsilateral side to injury (Guo et al., 2013).

Most studies demonstrating a role for mitochondria in neuropathic pain have focused on oxidative stress and the production of reactive oxygen species (ROS). Heat hyperalgesia in a chronic constriction injury (CCI) was inhibited using the antioxidants TEMPOL, N-acetylcysteine and tirilazad (Khalil et al., 1999; Tal, 1996; Wagner et al., 1998). In addition, to oxidative stress, mitochondrial fission has been implicated in models of neuropathic pain. Specifically, inhibiting dynamin-related protein 1 (Drp1), a GTPase involved in mitochondrial fission, reduced mechanical hyperalgesia caused by HIV/AIDS antiretroviral treatment or chemotherapy damage (Ferrari et al., 2011). Mitochondria are known to be affected be in EAE, however it is still unknown whether mitochondrial dysfunction is present within the dorsal horn of the spinal cord. We thus set out to gather preliminary data on whether the mitochondrial proteins involved in fission and fusion are disrupted within the dorsal spinal cord during early EAE. Our results show that at the onset of clinical signs of EAE, mitochondrial proteins are increased in the dorsal horn of the spinal cord. This increase was associated with a disruption of mitochondrial fission and fusion proteins.

5.1 METHODS

5.1.1 EAE Induction and Assessment

All animal studies were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with the approval from Animal Care and Use Committee: Health Sciences for the University of Alberta. EAE was generated in 10 to 12 week old female C57/BL6 mice (Charles River) (n= 40) using myelin oligodendrocytes glycoprotein (MOG) 35-55 obtained from Peptide Synthesis Facility at the University of Calgary. EAE was induced by subcutaneous injection bilaterally into the thigh of 50 μg of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1.5mg/mL. An intraperitoneal injection of 300 ng pertussis toxin (List Biological laboratories, Cedarlane, Canada) was administered on the day of induction and 2 days later. Mice were monitored daily and determined to be at clinical grade 1 (disease onset) when a flaccid or paralysed tail was first observed.

5.1.2 Histology and Immunocytochemistry

Whole spinal cord tissue samples were taken either when mice reached clinical grade 1 (flaccid, paralysed tail) or 35 days after induction of EAE (chronic EAE). Mice were euthanized by an overdose of Euthansol (340mg/ml) and then perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (transcardially). Lumbar spinal cords were removed, post-fixed overnight and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm). To assess mitochondrial content, a mouse anti-Cox IV (1:250, Abcam) and a mouse anti-Tom20 (1:50, Santa Cruz) were used. To localize the increase in mitochondrial

content, Tom20 was co-immunolabeled with anti-NeuN conjugate 488 (1:500, Chemicon), rat anti-GFAP (1:1000, Invitrogen), and rabbit anti-Iba-1(1:200, Wako). These antibodies were visualized using goat anti-mouse, goat anti-rat or goat anti-rabbit Alexa Fluor® 488 or 594 (1:200, Invitrogen-Life Technologies, Burlington ON). Twenty times magnification images of the spinal cord dorsal horn were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

5.1.3 Western Blots Analysis

Dorsal spinal cord samples were taken from mice that reached a clinical score of grade 1 after euthanasia with Euthanol and transcardial perfusion with 0.9% saline. These samples were flash frozen using liquid nitrogen. Protein was extracted using 10% RIPA buffer, diluted to a concentration of 1 μg/ml and placed at -80°C for storage. Dorsal spinal cord samples (20 μl) were separated on a 4-20% gradient SDS gels (Bio-Rad). The samples were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked using 5% BSA in PBS-Tween 20 (PBS-T) (0.05%), and then incubated overnight at 4°C in primary antibody: rabbit anti-Drp1 (1:250, Santa Cruz), anti-MFN2 (1:250, Santa Cruz) and mouse anti-Cox IV (1:500, Abcam) diluted in 1% BSA in PBS-T. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit (1:10,000, Jackson ImmunoResearch) or goat anti-mouse (1:10,000, Jackson ImmunoResearch). Membranes were re-probed with mouse anti-β-actin (1:20,000, Sigma) to ensure equal loading of samples. The binding of HRP-conjugated secondary was detected using the Amersham ECL western blotting analysis system (GE Healthcare). Membranes were imaged using a Bio-Rad gel dock system. Protein expression

levels were quantified using ImageJ software and were normalized to the loading control. Results are the relative expression of protein levels in EAE compared to CFA.

5.1.4 Statistical Analysis

Statistical analysis was carried out using the Student's t-tests and one-way ANOVAs with Tukey *post hoc* tests. For non-parametric data the Mann-Whitney Rank Sum test was used. Significance was set at $P < 0.05$. All tests were conducted using SigmaPlot 13 software.

5.2 RESULTS

5.2.1 Levels of mitochondrial proteins are increased at disease onset in EAE.

We have previously published that voluntary wheel running reduces the expression of mitochondrial proteins within the dorsal spinal cord at the onset of clinical sign in EAE (Benson et al., 2015) (see chapter 4). To gather preliminary evidence about the expression of mitochondrial protein in EAE we examined tissue sections taken at the onset of EAE clinical signs and at the chronic stages of EAE. Similar to our previous experiments, onset tissue samples were collected at the first signs of disease (clinical grade 1) and chronic samples were collected 30 days post EAE induction. At the onset of EAE the number of Tom20 positive cells within the dorsal horn are increased compared to CFA controls (Fig. 5.1 A, B). However, at the chronic stage, the levels Tom20 in the dorsal horn of the spinal cord tended towards CFA levels (Fig. 5.1 C). To corroborate the increases in Tom20, EAE spinal cord tissue sections were stained for a second mitochondrial marker, COX IV. Analogous to the Tom20 staining, we observed an increased expression of COX IV in the dorsal spinal cord of EAE mice at the onset

of clinical signs, and like Tom20, the levels of COXIV returned to CFA levels at the chronic time point (Fig. 5.1 D-F).

5.2.2 Increased Tom20 expression observed in EAE colocalizes with NeuN staining in the dorsal spinal cord

To determine the cell type contributing to the increased Tom20 expression at the onset of the clinical signs of EAE we conducted preliminary immunohistochemistry colocalization experiments with markers of microglia, astrocytes and neurons. We selected cell types thought to contribute to the hypersensitivity associated with EAE. Tom20 appeared not to colocalize with the microglial/macrophage, IBA1, in either CFA or EAE samples (Fig 5.2. A-F). Next we assessed whether Tom20 colocalized with the astrocyte marker GFAP. While Tom20 and GFAP were co-expressed, the amount of staining did not appear to change with EAE; making it unlikely that astrocytes are contributing to the increases in Tom20 (Fig. 5.3 A-F). However, the increased levels of Tom20 in dorsal spinal cord colocalized with the neuronal marker NeuN, suggesting that Tom20 expression in neurons is responsible for the elevated mitochondrial proteins in EAE (Fig. 5.4 A-F). These colocalization experiments are only a preliminary assessment of the cellular location of Tom20 expression.

5.2.3 Fission and fusion protein expression in the dorsal spinal cord

To further understand the cause of the increased mitochondrial protein expression, we isolated dorsal spinal cord tissue samples from the rest of the spinal cord for use in a Western blot analysis. The increased levels of Tom20 and COXIV, observed using immunohistochemistry, suggest an increase in overall mitochondrial number or mass. Mitochondrial fission and fusion are key pathways in controlling the amount and quality of

mitochondria. To assess whether mitochondrial fission was affected at the onset of EAE we measured the total amount of dynamin-related protein 1 (Drp1), a cytoplasmic mediator of mitochondrial fission. In addition, we also measured the levels of Drp1 phosphorylated at serine 616 (pDrp616). pDRP616 is an indicator of an active form of the enzyme (Taguchi et al., 2007). Western blot analysis revealed that Drp1 was increased at the onset time point within the dorsal spinal cord (log2 fold change, EAE: 2.59±0.52 SEM, CFA: 0±0.52, P=0.006) (Fig. 5.4 B). Interestingly, this increase in total DRP1 was associated with a decrease in the expression of pDrp616 in EAE mice (Fig. 5.4 C). In addition, we measured the expression of the mitochondrial protein FIS1, which binds Drp1 to the outer mitochondrial membrane. FIS1 levels remained unchanged between EAE at the onset of clinical signs and control CFA animals (Fig. 5.4).

We next asked whether EAE induced any changes in the proteins involved in mitochondrial fusion. Specifically, we measured the levels of mitofusin 2 (MFN2), which mediates the fusion of the outer membranes of adjacent mitochondria (Knott et al., 2008) . In tissue samples taken from the dorsal spinal cord, MFN2 was significantly decreased in EAE at the onset of clinical signs compared to CFA controls (log2 fold change, EAE: -4.28±2.10SEM, CFA: 0±0.69, P=0.009) (Fig. 5.3). MFN2 is partly under the control of PGC-1α, which is a master regulator of energy metabolism and mitochondrial biogenesis (Soriano et al., 2006). At the onset of clinical signs of EAE there was a significant decrease in PGC-1α compared to CFA control mice (Fig. 5.4). These western blot results indicate that in early EAE disease within the dorsal spinal cord, there is a deregulation of proteins involved in the fission and fusion of mitochondria.

5.3 DISCUSSION

Mitochondrial function has been suggested to have a role in many aspects of MS disease including susceptibility, active lesion formation, white and grey matter damage and neurodegeneration (Witte et al., 2014). Using a model of active CNS inflammation, EAE, we found a number of significant changes in mitochondrial protein expression within the dorsal spinal cord of EAE animals. In particular, there was a large increase in the mitochondria fission protein Drp1 and decreases in MFN2 and PCG-1α expression. These results suggest that early in EAE there is a significant disruption of mitochondrial function and dynamics. These mitochondrial changes were observed in the dorsal spinal cord, an area important for spinal pain processing, adding weight to the suggestion that mitochondrial damage may contribute to sensory dysfunction in EAE.

Although it is known that mitochondrial transport is disrupted in EAE, there has been very little work investigating whether mitochondrial fission and fusion are affected in EAE. Therefore, we investigated the expression of the mitochondrial fission protein Drp1 and the fusion protein MFN2. We found that in the dorsal spinal cord of EAE mice there was a significant increase in the levels of Drp1 (fission) and a decrease in the level of MFN2 (fusion). These results alone would suggest an overall increase in mitochondrial fission. However, Drp1 is heavily regulated by post-translational modifications including phosphorylation, ubiquitination, s-nitrosylation and SUMO-ylation (Braschi et al., 2009; Cho et al., 2009; Figueroa-Romero et al., 2009; Karbowski et al., 2007; Wang et al., 2011). Specifically, phosphorylation at serine 616 promotes Drp1 activity, while phosphorylation at a separate site, serine 637, decreases activity (Chang and Blackstone, 2007; Taguchi et al., 2007). Western blot analysis of the dorsal spinal cord found reduced levels of pDrp616 in EAE mice, which would

suggest a decrease in Drp1 activity. Future research should examine the amount of Drp1 phosphorylated at ser637. In EAE, an increase in the phosphorylation of Drp1 at ser637 would indicate that the build up of Drp1 in the dorsal spinal cord is not mediating fission. In this scenario the increased Drp1 could simply be a marker of mitochondrial dysregulation with no functional consequence or the increased Drp1 could be promoting action unrelated to mitochondrial fission. However, in EAE, if ser637 is also not being phosphorylated, it is possible that Drp1 can mediate fission due to the lack of ser637 repression (Chang and Blackstone, 2007). Taken together, these results reveal significant alterations in expression and regulation of mitochondrial proteins in the dorsal spinal cord during EAE.

Drp1 has been implicated in several non-mitochondrial processes, such as synaptic plasticity and synaptic vesicle endocytosis (Li et al., 2013; Li et al., 2004). Expression of mutant Drp1 in primary hippocampal neurons reduced the number of dendritic spines, presynaptic boutons and post-synaptic markers (Li et al., 2004). Knockout and knockdown of Drp1 also showed similar changes in synaptic markers (Ishihara et al., 2009; Wang et al., 2009). In addition, *in-vivo* Drp1 overexpression enhanced adult hippocampal neurogenesis and memory (Steib et al., 2014). Li et al. (2013) described that Drp1 can be recruited to synaptic vesicle membranes by mitochondrial fission factor (MFF) where Drp1 complexes with Bcl-xl. This binding of DRP1 to Bcl-xl enhanced synaptic vesicle uptake and increased the vesicle recovery pool (Li et al., 2013). It is unclear whether elevated Drp1 levels can affect this process and whether the effect of Drp1 at the synapse is dependent on the phosphorylation at ser616 or ser637. However, synaptic changes are known to underlie several neuropathic pain conditions. Therefore, it is possible that the increased levels of Drp1 in the dorsal spinal cord could disrupt normal synaptic activity, thereby contributing to abnormal sensory function in EAE.

Opposing the activity of Drp1 are the mitofusin proteins. MFN1 and MFN2 are present in mammalian cells and are critical for mitochondrial fusion. Deletion of MFN1 or MFN2 significantly reduces fusion and deletion of both proteins prevents fusion entirely (Chen et al., 2003). In addition, the mitofusins are involved in several other cellular processes, such as metabolic homeostasis, cell signalling, cell proliferation and mitochondrial transport (Chapman et al., 2013; Chen et al., 2014a; Mourier et al., 2015; Tubbs et al., 2014). Mutations in MFN2 manifest clinically as a subpopulation of Charcot-Marie-Tooth disease (CMT) (Verhoeven et al., 2006). Specifically, MFN2 mutations occur in the axonal variant CMT2A, which is characterized by muscle weakness, hyporeflexia and sensory loss in the lower limbs (Stuppia et al., 2015). It is interesting that while the MFN2 mutations are present in all cells, the pathology of CMT2A is restricted to the peripheral nervous system. This suggests that these nerves are less able to compensate for the loss of MFN2. Our present study only goes as far to suggest a dysregulation of mitochondrial dynamics and does not address the functional consequence of decreased MFN2 in the dorsal spinal cord. Further studies are required to determine whether the loss of MFN2 is involved in disease progression or the pain hypersensitivity associated with EAE.

Mitochondrial function and dynamics can also be controlled at the transcriptional level. The transcription factor, PGC-1α, is an important regulator of mitochondrial biogenesis, oxidative metabolism, antioxidant pathways and mitochondrial dynamics (Chaturvedi and Flint Beal, 2013). In addition, PGC-1α is known to act as a coactivator along with estrogen-related receptor alpha (ERRα) to bind the MFN2 promoter and enhance transcriptional activity (Soriano et al., 2006). We observed a decrease expression of PGC-1α at the onset of the clinical signs of EAE. This suggests that mitochondrial biogenesis is not contributing to the increased mitochondrial content in the dorsal horn of the spinal cord. However, decreased PGC-1α likely

contributes to EAE pathology, as two studies have investigated the role of PGC-1α in MS. Firstly, Witte et al. (2011) described reduced expression of PGC-1α in cortical neurons of MS patients and this decrease correlated with areas of neuronal loss (Witte et al., 2013). The cortical decrease in PGC-1α levels was also associated with reduced mitochondrial antioxidants (Witte et al., 2013). Secondly, the same authors found elevated PGC-1α expression in astrocytes within active MS lesions (Nijland et al., 2014). From *in vitro* experiments, Nijland et al., 2014 suggest that PGC-1α overexpressing astrocytes are more resistant to the presence of high ROS and produce less Il-6 and CCL2 (Nijland et al., 2014).

In our current study, it is unclear whether the loss of PGC-1α is in neurons or glial cells. Furthermore, although our tissue samples are taken at the onset of the clinical signs of EAE, which represents a period of active inflammation, these samples contain a mix of spinal cord grey and white matter. A more specific analysis of EAE tissue is required to determine if there is a difference between PGC-1α expression in the dorsal column white matter compared to the superficial laminae of the spinal cord. In addition, PGC-1α is known to be affected by exercise (Oliveira et al., 2014), suggesting that changes in PGC-1α levels could partially underlie the beneficial effect of exercise on EAE associated pain. Specifically, after exercise muscle tissue expresses greater PGC-1α that can lead to changes in thermoregulation and reduced muscle inflammation (Bostrom et al., 2012; Eisele et al., 2015).

Impaired PGC-1α functions have also been implicated in several other neurodegenerative disorders, including Huntington's, Parkinson's and Alzheimer's (Chaturvedi et al., 2009; Mudo et al., 2012; Qin et al., 2009; St-Pierre et al., 2006) . In addition, the overexpression of PGC-1α can be protective in these models of neurodegenerative disease. For example, transgenic overexpression of PGC-1α in dopaminergic neurons induces resistance against the neurotoxin

MPTP (Mudo et al., 2012). In addition, these authors found that the natural antioxidant compound resveratrol could limit MPTP-induced neuronal loss (Mudo et al., 2012). In Alzheimer's models, increasing the expression of PGC-1α protected cells against mutant α-synuclein (Zheng et al., 2010).

Mitochondrial changes are known to occur early in EAE disease progression; in fact dysfunctional mitochondria can be detected prior to the infiltration of T-cells into the CNS (Qi et al., 2006). Similarly, we observed changes in mitochondrial protein expression very early in the EAE disease course. Although these changes can likely occur throughout the CNS, we focused on the dorsal horn of the lumbar spinal cord, an area critical for the processing of sensory signals. We observed significant changes in mitochondrial protein expression and regulation, which could affect a wide array of cellular processes including energy demands, calcium storage, ROS production, mitochondrial motility and apoptosis (Bertholet et al., 2015). Disruption of any of these processes could contribute to the pain phenotype in EAE. Mitochondrial function appears to be uniquely sensitive to the initial inflammatory insult, further work is required to determine whether changes in mitochondrial fusion and fusion proteins underlie symptom progression in EAE.

5.4 FIGURES

Figure 5.1

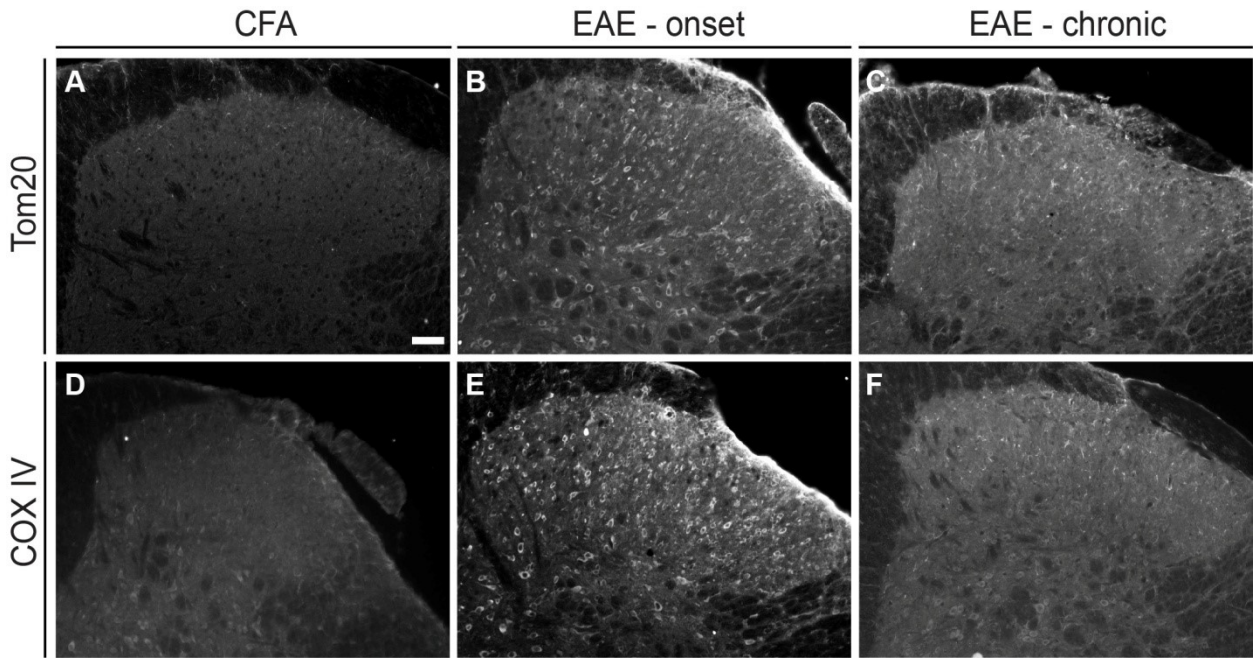

Figure 5.1: Mitochondrial protein expression increases at the onset and chronic EAE disease. Representative 20x images of Tom20 and Cox IV staining in spinal dorsal horn from a CFA control mouse (A, D), an EAE mouse at the onset of clinical signs (B, E) and an EAE mouse 30 days post induction (C, F). Within the dorsal spinal cord both Tom20 and COX IV appeared to increase at the onset of EAE disease, but are no longer elevated during chronic disease. Scale bar = 250μm and applies throughout.

Figure 5.2

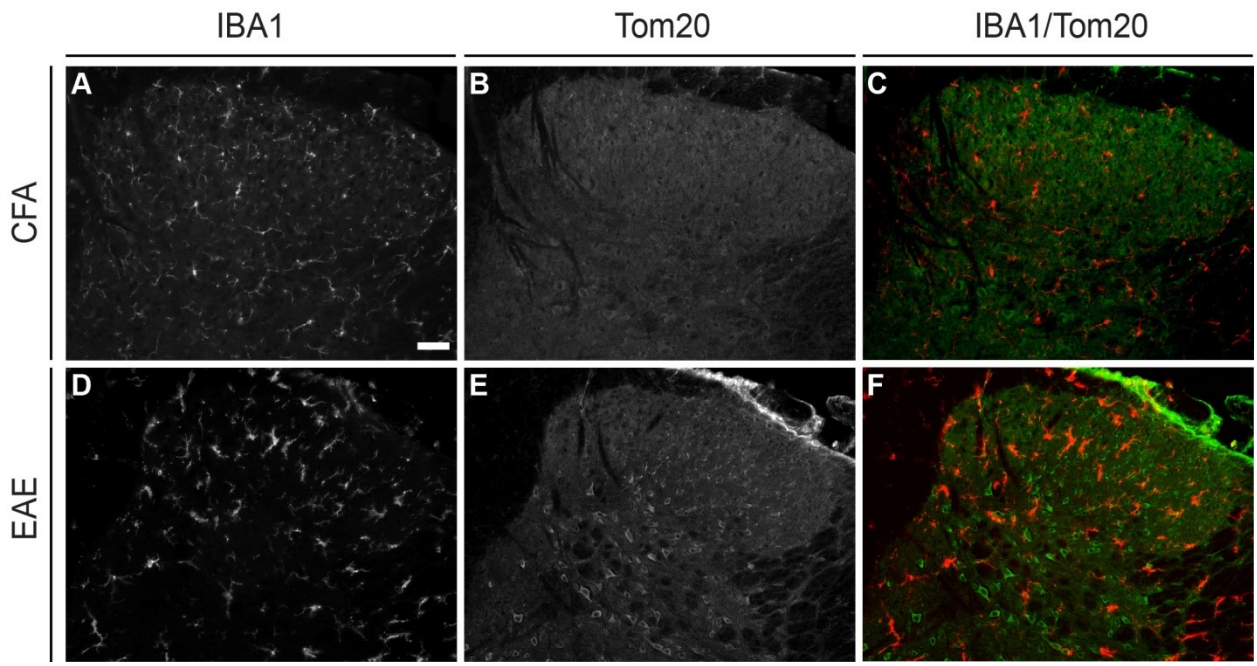

Figure 5.2: Increased Tom20 expression observed in EAE is not colocalized with IBA1 staining in the spinal dorsal horn. Representative 20x images of spinal dorsal horn of CFA control (A-C) and EAE onset (D-F) visualized for IBA1 (microglia/macrophages) (A, D) and Tom20 (B, F). IBA1 does not colocalize with Tom20 in the dorsal horn of the spinal cord. Scale bar in (A)= 250μm and applies throughout.

Figure 5.3

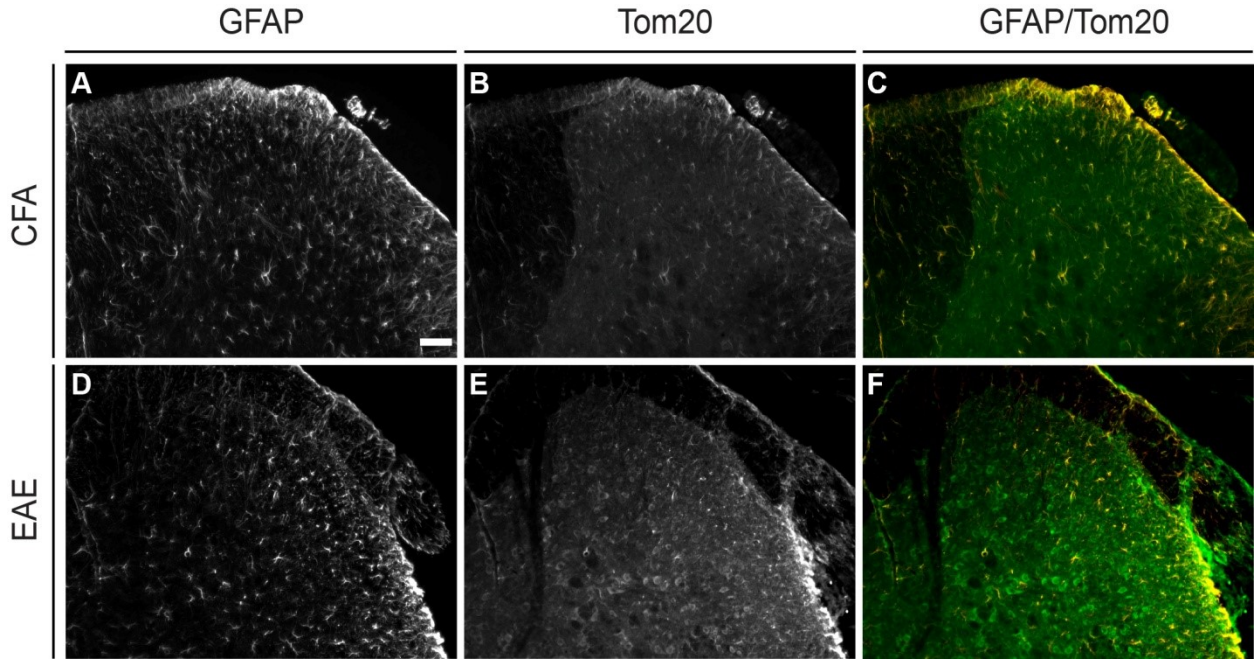

Figure 5.3: Increased Tom20 expression observed in EAE is not colocalized with GFAP staining in the spinal dorsal horn.

Representative 20x images of spinal dorsal horn of CFA control (A-C) and EAE onset (D-F) visualized for GFAP (astrocytes) (A,D) and Tom20 (B, F). Tom20 colocalized with GFAP staining, but is not the source of the increased Tom20 in the dorsal spinal cord. Scale bar in (A)= 250μm and applies throughout.

Figure 5.4

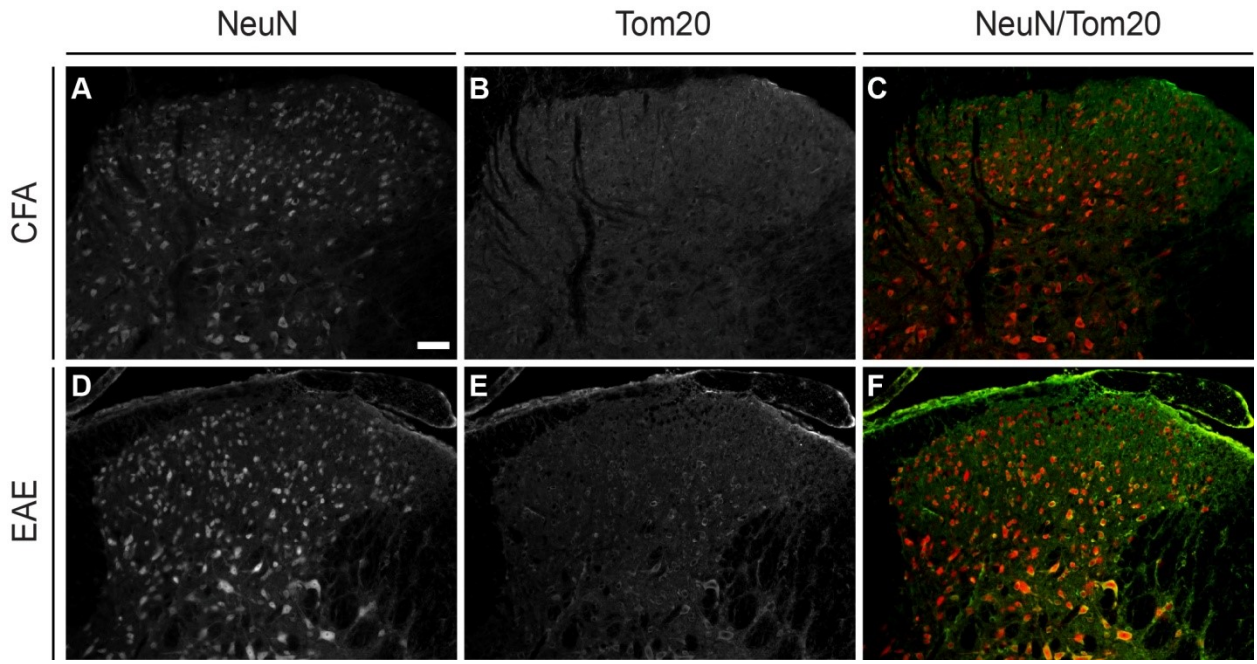

Figure 5.4: Increased Tom20 expression observed in EAE is not colocalized with Tom20 staining in the spinal dorsal horn.

Representative 20x images of spinal dorsal horn of CFA control (A-C) and EAE onset (D-F) visualized with NeuN (neurons) (A, D) and Tom20 (B, F). NeuN colocalized with the increased dorsal horn Tom20 expression observed in EAE at the onset of clinical signs. Scale bar in (A)= 250μm and applies throughout.

Figure 5.5

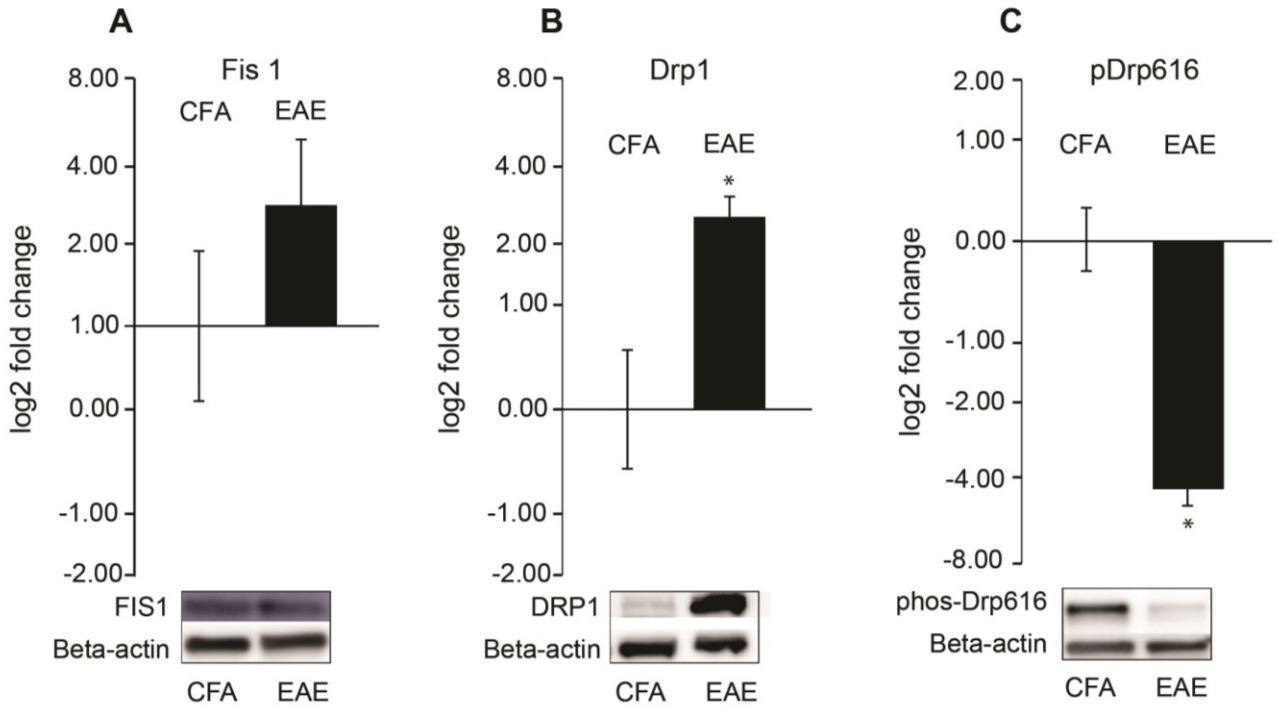

Figure 5.5: Changed expression and dysregulation of mitochondrial fission proteins in the dorsal spinal cord. (A-C) Protein levels normalized to CFA controls. (A) Fis1 expression is not significantly different at the onset of EAE compared to CFA controls. (B) A mediator of mitochondrial fission, DRP1, (dynamin-related protein 1) is significantly increased during the onset of EAE compared to normalized CFA levels. (C) Compared to CFA controls EAE mice at the onset of clinical sign have significantly decreased levels of phos-Drp616 (*$P<0.05$, Student's t-test). Error bars in (A-C) represent ±SEM.

Figure 5.6

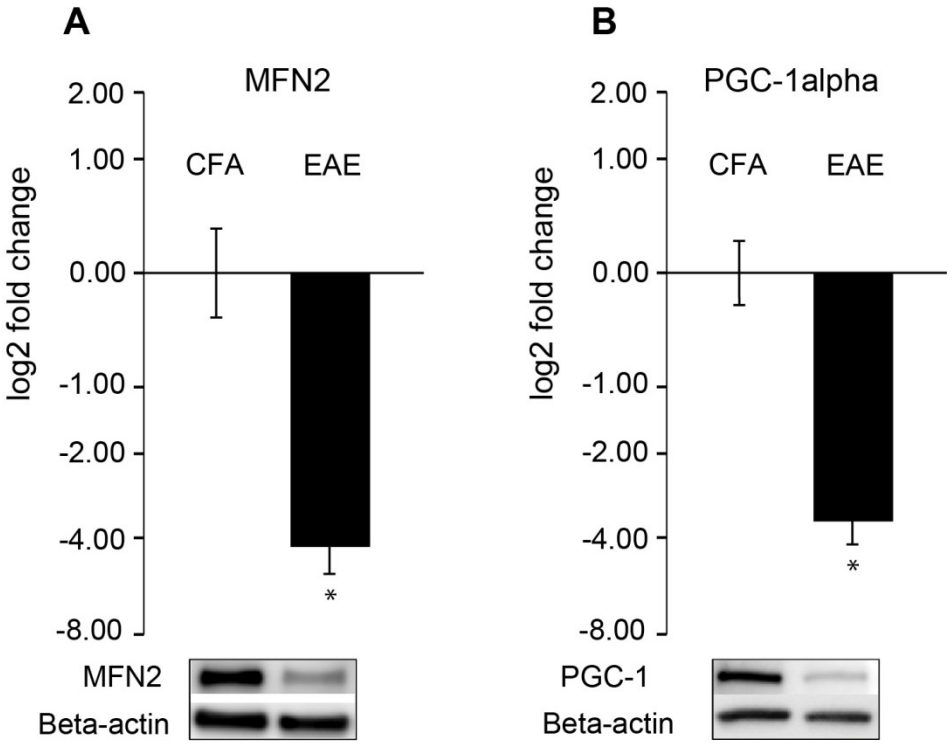

Figure 5.6: Reduced expression and dysregulation of mitochondrial fusion proteins in the dorsal spinal cord.

(A-B) Protein levels normalized to CFA controls. (A) A mediator of mitochondrial fusion, MFN2, is significantly decreased during the onset of EAE compared to normalized CFA levels. (B) Compared to CFA controls EAE mice at the onset of clinical disease signs have significantly decreased levels of PGC-1α (*$P<0.05$, Student's t-test). Error bars in (A-B) represent ±SEM.

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Chapter 6

General Discussion

6.1 DISCUSSION 1: NEUROTRANSMITTERS IN THE EAE MODEL

6.1.1 Neurotransmitters in EAE

Multiple sclerosis (MS) is associated with a significant disruption in several key neurotransmitter systems. There is an increasing amount of research indicating that reductions in these neurotransmitter levels contribute to both the peripheral and central components of MS pathology. Chapters 2 and 3 focused on examining the effects of the antidepressant phenelzine (PLZ) as a treatment for EAE. Treating EAE mice with PLZ at day 7 post-induction delayed the onset of clinical signs and lessened disease severity in the chronic phase (chapter 2). PLZ given at day 7 also improved exploratory behaviours in the open field and increased the score of activity/attention. To test PLZ in a more clinically relevant situation, the next set of experiments examined the outcome of beginning PLZ treatment at first clinical signs of disease (chapter 3). When PLZ was given after the establishment of these clinical signs, it was still effective at improving the EAE symptoms. Overall, the work reported in chapters 2 and 3 suggests that using this MAO inhibitor and GABA-T inhibitor to restore the levels of several neurotransmitters simultaneously improves EAE and possibly has therapeutic potential for MS.

6.1.2 PLZ and PEH and GABA

The combined work of chapters 2 and 3 suggests that increased levels of GABA are critical for the effect of PLZ in EAE. When PLZ treatment was started at day 7 it was evident that the improvement in open field behaviours was not sustained for the duration of the experiment. After 30 days of PLZ treatment, both the activity/attention score and the number of line crossings in the open field were the same as untreated controls. However, on day 37 there was an improvement in clinical score and the rotorod assay, both measures of locomotor function.

Interestingly, levels of 5-HT and NE were significantly elevated at the end of the experiment but GABA was not. This suggests that increased GABA is required to improve anxiety/sickness behaviours seen in the open field assay. Drugs that target the GABAergic pathways are also known to have anxiolytic properties. Thus it is not surprising that although PLZ was originally developed as an antidepressant, it can also be used to treat social anxiety disorder (Song et al., 2013).

Future experiments should be conducted to determine whether the specific EAE-related behavioural deficits are related to individual neurotransmitter systems. This can be accomplished by using several derivatives of PLZ: phenylethylidenehydrazine (PEH) and N^2-acetyl-PLZ (N2-Ac-PLZ). PEH is an active metabolite of PLZ that potently inhibits GABA-transaminase (GABA-T) but has minimal effects on MAO activity (Parent et al., 2002). Therefore, PEH significantly elevates GABA levels in the CNS but has no effect on 5-HT or NE (Duffy et al., 2004). N2-Ac-PLZ is a derivative of PLZ that inhibits MAO but does not affect GABA-T (McKenna et al., 1994). Treating mice with N2-Ac-PLZ elevates the levels of 5-HT and NE but does not affect GABA levels. Comparing the effect of these drugs on EAE will help to differentiate the role of GABA, norepinephrine and serotonin in the motor and non-motor symptoms associated with EAE.

6.1.3 PLZ and Dopamine in the Spinal Cord

In chapter 3, in addition to the increases in 5-HT, NE and GABA we observed that PLZ given every second day drastically elevated the levels of dopamine (DA) in the spinal cord. In fact, in untreated EAE animals, DA was below the limits of detection of the HPLC method. Chronic treatment with PLZ also lead to higher levels of DA in the brain and spinal cord (data not

published). This is not surprising as the effects of PLZ on DA result from the inhibition of MAO B and are not related to the production of metabolite PEH (Al-Nuaimi et al., 2012). In both the chronic PLZ treatment condition and when PLZ treatment began "at onset", PLZ treatment elevated DA levels in both the spinal cord and the brain.

DA is distributed throughout the CNS; however, the concentration of DA in the brain is much higher than that in the spinal cord. The main source of spinal DA is from descending fibers projecting from hypothalamic nuclei (Qu et al., 2006; Sharples et al., 2014). However, based on experiments with complete spinal cord transections, there may be a small group of interneurons capable of DA synthesis in the spinal cord (Hou et al., 2015). Further experiments are required to determine whether PLZ is enhancing DA from descending fibres or locally within the spinal cord. However, due to the strong effect of PLZ on DA in the spinal cord (40x increase) it is worth discussing the possible consequences of DA on the EAE model.

The majority of the work examining the role of DA in MS has focused on DA's effect on immune regulation. Various immune cells can respond to exogenous DA through their expression of various DA receptors. Lymphocyte and dendritic cell populations also contain the enzyme tyrosine hydroxylase and therefore produce DA endogenously (Cosentino et al., 2002a; Cosentino et al., 2002b). The effect of DA on the immune response is likely context-dependent. In resting or naive T-cells, DA can promote the expression of adhesion molecules and promote the secretion of either TNF-α or IL-10 (Besser et al., 2005; Levite et al., 2001). In contrast, T-cells taken from healthy people and activated with anti-CD3 or IL-2 are inhibited by the presence of DA (Saha et al., 2001; Sarkar et al., 2006).

There is evidence that immune cells from MS patients respond inappropriately to exogenous DA. Peripheral blood mononuclear cells (PBMCs) from untreated patients with

RRMS showed lower amounts of the DA receptor D_5 and the proliferation of these cells was no longer inhibited by DA (Giorelli et al., 2005). PBMCs isolated from patients treated with IFN-β showed a normal response to DA and reduced levels of the DA receptor D_3 (Giorelli et al., 2005). More recent studies have indicated that DA plays a role in the expansion of TH17 cells in MS. Blocking DA using a D1-like receptor antagonist inhibited Th17 differentiation and improved symptoms in the EAE model (Nakano et al., 2008). However, the same paper showed that blocking D2-like receptors induced dendritic cell-mediated Th17 differentiation (Nakano et al., 2008). A 2014 paper by Ferreira et al. suggested that DA amplified the Th17 phenotype in MS patients possibly through decreases in the levels of cytokines produced by Tregs (Ferreira et al., 2014). In contrast, treating EAE mice with the D2/D3 receptor agonist immediately after immunization completely prevented the development of motor deficits in EAE (Lieberknecht et al., 2016). These studies highlight the fact that it is difficult to assign DA a positive or negative value in the context of MS. Using this framework in an attempt to understand the effects of PLZ in EAE is even more difficult. PLZ causes broad increases in 5-HT, NE, GABA levels and possibly has direct effects on immune cells. In combination with the other broad effects of PLZ, it is difficult to determine whether increased levels of DA are pro- or anti-inflammatory.

6.1.4 Direct effects of PLZ on Immune cells

An alternative mechanism for the effect of PLZ in EAE could be related to the direct interaction of PLZ with non-neuronal cells. Our lab has started preliminary experiments exploring the outcome of culturing human PBMCs with PLZ. In these experiments PBMCs were isolated from healthy donors and activated using anti-CD3 stimulation. The PBMCs were then exposed to 10μM, 100μM and 300μM concentrations of PLZ during the 72-hour activation period with anti-

CD3. As a marker of immune cell activation, the culture media was assessed for the amount of IFNγ. In these experiments, PLZ dose dependently decreased the amount of IFNγ produced during the 72 hour activation period. These results suggest that PLZ can have a direct effect on immune cell function. Part of this effect is possibly due to PLZ inhibiting MAO activity in these immune cells, as human PBMCs express MAO-A on the outer mitochondrial membrane (Chaitidis et al., 2004). The Th-2 cytokines IL-4 and IL-13 also cause an up-regulation of MAO-A, indicating that MAO-A may be involved in immune regulation (Chaitidis et al., 2004). In addition, the presence of MAO-A in PBMC cultures could allow for the production of PEH, as both macrophages and T-cells contain the enzyme GABA-T (Bhat et al., 2010). Treating macrophages with two irreversible GABA-T inhibitors, vigabatrin and gabaculine, reduces IL-1β and IL-6 expression (Bhat et al., 2010). In the future, experiments should be conducted to assess the outcome of treatment with PEH or N2-acetyl-PLZ on PBMC cultures. This will permit the differentiation of the consequences of inhibiting MAO or GABA-T on immune cell activation.

6.1.5 Serotonin and melatonin

Recent evidence has associated the hormone melatonin with MS disease. Melatonin is produced by the pineal gland and levels are elevated at night (Emens and Burgess, 2015). Melatonin is best known for regulating circadian patterns, however it is also an effective anti-oxidant and a possible immune-modulator (Emens and Burgess, 2015; Kashani et al., 2014). Incidentally, there are several clinical observations that suggest a role for melatonin in MS. MS is associated with abnormal sleep and at night people with MS build up less 6-sulphatoxymelatonin, a melatonin metabolite, in their urine (Melamud et al., 2012). Recently, it was observed that melatonin contributes to the seasonal variability of MS relapse rate (Farez et al., 2015). Using a cohort of

139 people with MS, Farez et al. (2015) found that melatonin levels peak in autumn/winter and that this peak was correlated with a reduced relapse rate. This finding provides some clarity to the observation that MS relapses rates are greatest in the spring and summer, which seems paradoxical considering that (supposedly anti-inflammatory) vitamin D levels are greatest in this period (Spelman et al., 2014). Furthermore, treating EAE mice with melatonin suppressed the disease and was associated with decreases in IL-17 cells and IL-10 producing T-cells (Farez et al., 2015). This work indicates that melatonin can have a potent effect on EAE through its immune modulatory properties.

In light of the apparent role of melatonin in EAE, it is worth considering whether any of the beneficial effects of PLZ can be attributed to changes in melatonin. In our experiments the overall contribution of this mechanism may be very small, as the strain of mice used in our experiments (C57/BL6) is considered to be pineal melatonin deficient. The pineal gland of C57BL/6 mice expresses a mutant form of AANAT; the enzyme required to make melatonin. However, studies suggest that in C57Bl/6 mice there are extra-pineal sources of melatonin that create a low concentration in the serum. In particular, several immunological tissues, including the spleen and thymus, are capable of synthesizing melatonin (Gomez-Corvera et al., 2009).

Melatonin is synthesized from serotonin and is degraded through various pathways that include MAO A (Hardeland, 2010). Therefore, it is possible that PLZ could influence the levels of melatonin by preventing the breakdown of both the melatonin precursor (serotonin) and melatonin itself by inhibiting MAO A. This idea is indirectly supported by studies that show that when depressed patients are treated with the MAO A inhibitors, clorgyline and tranylcypromine, their plasma levels of melatonin are increased (Murphy et al., 1986). In the EAE model, PLZ

treatment could lead to increased levels of melatonin and improve the disease through its immune modulatory and antioxidant mechanisms.

6.1.6 Summary

In chapters 2 and 3 we have demonstrated that EAE disease can be improved using the MAO inhibitor PLZ. Both behavioural and pathological improvements were observed by restoring the levels of 5-HT, NE, DA and GABA. PLZ treatment was effective both when given prior to or at the onset of clinical signs. Further work is required to determine the exact mechanisms and location of action, which will be difficult as beneficial effects of PLZ are likely a consequence of a combination of multiple mechanisms.

6.2 DISCUSSION 2: EXERCISE, PAIN AND MITOCHONDRIA IN EAE

6.2.1 Exercise and EAE

Although not always thought to be the case, exercise therapy is safe for patients with MS (Heine et al., 2015) . However, it has been difficult to determine the best type, timing and amount of exercise therapy for patients with MS. Furthermore, even less is known about the effects of exercise on the cellular pathology of MS. The experiments in chapter 4 set out to determine whether a limited amount of daily exercise could improve pain hypersensitivity associated with EAE. Our work showed that 1 hour per day of voluntary wheel running modestly improved pain in EAE mice. This improvement was associated with reduced inflammatory pathology in the dorsal spinal cord and less ongoing oxidative stress. Encouraged by these results, there are several possible future directions for this project.

6.2.2 Exercise in Male and Female Mice with EAE

The experiments in chapter 4 were done using only female animals. This is fairly common for EAE experiments and is somewhat justified because the prevalence of MS in females is significantly greater than males (Marrie et al., 2016). However, it is becoming more and more obvious that males and females can have distinct disease mechanisms. Thus an effective therapy in one sex may less effective in the other. This may also be the case for exercise therapy in MS. In healthy people, exercise can affect males and females differently. During exercise females oxidize more lipids and less carbohydrate, a difference attributed to estrogen levels (Carter et al., 2001). The effects of exercise on the heart muscle tissue can differ also between sexes (Dworatzek et al., 2014). In addition there is some evidence that exercise can affect immune cell populations and cytokine levels differently in males and females (Gillum et al., 2011). These studies highlight the importance of investigating exercise in both male and female mice with EAE. In particular this is an important consideration when studying pain in MS, as there are known to be distinct pain pathways in females and males (Sorge et al., 2015). Therefore in our case, both the treatment and behavioural outcome can vary between the sexes, making it far from a guarantee that voluntary wheel running will improve the pain in male EAE animals.

6.2.3 Exercise and Oxidative stress

In addition to its effect on inflammatory markers, we also observed that exercise significantly reduced oxidative stress and mitochondrial protein expression in the dorsal spinal cord. Short term and chronic exercise is known to influence reactive oxygen species (ROS) levels within the body. This effect on ROS is dependent on the type and duration of the exercise. Acute and strenuous exercise transiently increases the generation of ROS (Bloomer and Fisher-Wellman,

2008). However, this increase is accompanied by the activation of antioxidant pathways. It has been suggested that activity of the antioxidant enzyme, superoxide dismutase (SOD), is higher in trained athletes, which underlies the general observation that oxidative stress is lower in athletes than in sedentary people (Bloomer and Fisher-Wellman, 2008; Ortenblad et al., 1997). It is possible that 1 hour per day of voluntary wheel running enhanced the activity of antioxidant enzymes in EAE. However, both infiltrating immune cells and resident glial cells are a significant source ROS in EAE. Therefore, the effect of daily wheel running on the redox state in EAE could also be a result of a reduction in T-cell number and microglia activation. Future studies should compare the amount of SOD activity to NADPH activity to determine whether 1 hour per day of exercise is promoting anti-oxidant pathways or reducing the production of ROS.

In addition to increased oxidative stress we also observed reduced expression of COXIV and Tom20 in the dorsal spinal cord with daily wheel running. These reductions were associated with less pain hypersensitivity in EAE. This suggested that changes in mitochondrial protein expression may contribute to pain in MS and EAE. This observation led to the experiments in chapter 5 and the ongoing work in our lab to investigate the role of mitochondrial dysfunction in pain hypersensitivity in EAE.

6.2.4 Mitochondria and EAE

Mitochondrial dysfunction has been well established in experimental models of MS and is suggested to have a role in the neurodegenerative processes of the disease (Witte et al., 2014). The work done in chapter 5 aimed to further investigate the possible mechanisms underlying the increases in Tom20 and COXIV observed in chapter 4. We observed that the increase in COXIV and Tom20 expression in the dorsal horn of the spinal cord was localized in neurons. Next we set

out to characterize several pathways involved in the regulation of mitochondrial number. Specifically, we examined proteins involved in the regulation of mitochondrial fission, fusion and biogenesis. This work revealed significant changes in expression and dysregulation of mitochondrial proteins in the dorsal spinal cord of EAE mice. The functional implication of these results is still unknown. Future experiments manipulating the expression and activity of these mitochondrial proteins is required to determine whether these changes contribute to behavioural deficits associated with EAE.

6.2.5 Drp1 and EAE

The GTPase activity of DRP1 is known to be critical for mitochondrial fission, and increasing DRP1 activity promotes mitochondrial fragmentation (Bleazard et al., 1999; Otsuga et al., 1998). To further investigate the role of Drp1 in EAE, I propose treating mice with EAE with the mitochondrial fission inhibitor mdivi-1daily. Mdivi-1 is known to block mitochondrial fission through the inhibition of Drp1 GTPase activity (Cassidy-Stone et al., 2008). Treating EAE mice with mdivi would reveal whether the increased Drp1 levels in the dorsal spinal cord contribute to the symptoms associated with EAE. Given the localization of the mitochondrial changes to the dorsal spinal cord, I would test both thermal and mechanical nociception in EAE treated with mdivi to assess whether Drp1 activity is related to pain in EAE.

In neuropathic pain there is a significant disruption of the sensory processing in dorsal horn of the spinal cord. Several synaptic mechanisms can underlie this dysfunction, including changes in afferent input, A-fibre sprouting, loss of c-fiber terminals and activity dependent plasticity (Luo et al., 2014). In MS and in the EAE model it is possible that increased Drp1 expression is related to these synaptic pain mechanisms. Expression of a dominant negative form

of DRP1 decreases mitochondrial targeting to the synapse and prevents spine formation (Dickey and Strack, 2011; Li et al., 2008). In addition, independent of mitochondria, DRP1 can associate with BCL-xl and clathrin in the synapse to form complexes that regulate vesicle exo/endocytosis, thereby influencing synaptic activity (Li et al., 2013). Based on these studies it is possible that mdivi-1 might be effective in reversing pain hypersensitivity in EAE independent of its effects on mitochondrial fission by normalizing synaptic function.

6.2.6 MFN2 and EAE

The levels of MFN2 were measured to determine whether mitochondrial fusion was disrupted in the dorsal spinal cord of EAE animals. We found that at the onset of EAE clinical signs, there was a significant decrease in MFN2 protein expression. Coupled with the elevated DRP1 levels, this indicates that within the dorsal spinal cord there is a significant dysregulation in the proteins involved in mitochondrial function. Despite this, MFN2 has several roles of independent of mitochondrial fusion. MFN2 is needed to tether mitochondria to the endoplasmic reticulum and this close interaction facilitates calcium signalling between the two organelles (de Brito and Scorrano, 2008). Disruption of mitochondrial calcium signalling could lead to changes in bioenergetics, apoptosis and the removal of damaged mitochondria, a process known as mitophagy (Finkel et al., 2015). In addition, mitochondrial calcium has been associated with TRPV1 activation and glutamate release (Medvedeva et al., 2008). Therefore, restoring the levels of MFN2 in EAE could modulate sensory function. MFN2 is also involved in anterograde transport of mitochondria in neurons (Misko et al., 2010). MFN2 links mitochondrial to the Miro/Milton complex that binds to kinesin-1 allowing movement along microtubules. Therefore, lower levels of MFN2 could contribute to the pathology of EAE through various mechanisms.

There are several compounds that can enhance mitochondrial fusion. Wang et al., (2012) found the small molecule compound, hydrazone M1, could promote mitochondrial elongation in cells with deletions of MFN1 or MFN2 (Wang et al., 2012). Interestingly this compound had no effect in WT or in MFN1/MFN2 double knockouts, suggesting that hydrazone M1 is selective for cells with fragmented mitochondria that have some capacity for fusion (Wang et al., 2012). In addition, the authors showed that hydrazone M1 could prevent 1-methyl-4-phenyl-pyridinium (MPP+)-induced cytotoxicity of dopaminergic neuroblastoma cells (Wang et al., 2012). The work done in chapter 5 found that within EAE spinal cords there is a loss of MFN2 expression, possible indicating a decrease in fusion. Instead of blocking mitochondrial fission, treating EAE mice with hydrazone M1 would allow us to determine whether enhancing fusion would improve EAE disease and secondary symptoms such as pain hypersensitivity.

6.2.7 Mitochondria, EAE and MAO

There is a possible link between PLZ, ROS and mitochondrial function.

ROS levels within a cell are kept in check by a complex antioxidant defense system. The most well studied and efficient system is the removal of ROS by superoxide dismutase (SOD) and peroxidases. SOD catalyzes the conversion of O_2^- to H_2O_2 that is then reduced to water by peroxidases, such as catalase and glutathione peroxidases (Flohe and Ursini, 2008). A breakdown in this process can lead to an uncontrolled build-up of O_2^- and H_2O_2, causing oxidative damage within the cell (Kokoszka et al., 2001). In addition to the electron transport chain, other sites on mitochondria can produce significant amounts of ROS. More specifically, the normal reaction catalyzed by MAO produces both H_2O_2 and reactive aldehyde intermediates. In fact the MAO can produce 48 fold more H_2O_2 than the ETC (Hauptmann et al., 1996). While

under normal conditions the cell may be able to cope with the H_2O_2 produced by MAO, in MS and in the EAE model, the addition of ROS from infiltrating T-cells, macrophages and mitochondrial damage likely overwhelms the SOD and peroxidase reactions. Lowering H_2O_2 produced by MAO enzymes has been considered as a method to reduce the overall amount of ROS (Kunduzova et al., 2002; Maurel et al., 2003). Specifically, blocking MAO function using MAO inhibitors like PLZ can reduce H_2O_2 with in a cell (Simonson et al., 1993). Therefore, it may be worth conducting further experiments with PLZ to determine whether any of the benefits observed in chapters 2 and 3 were due to changes in ROS production and mitochondrial function.

6.2.8 Summary

The work in chapter 4 demonstrated that a limited amount of daily exercise could improve pain hypersensitivity in EAE. This improvement was associated with reduced inflammation and less oxidative stress. Unexpectedly, exercise also decreased the expression of mitochondrial proteins in the dorsal horn of the spinal cord. This finding led us to further investigate proteins involved in mitochondrial fission and fusion in chapter 5 of this thesis. It appears that within the spinal cord of EAE animals there is a significant dysregulation of mitochondrial protein expression. Developing therapies that target Drp1, MFN2, PGC-1α and/or ROS production may restore mitochondrial function and that leads functional improvement in EAE and MS.

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