Exploring the role of sex differences in chronic pain

by

Katherine Ann Mifflin

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Neuroscience

University of Alberta

Abstract

Sex traditionally has been an understudied variable in the chronic pain literature. However, more recent research has begun to explore the role of sex in the etiology of chronic pain. The present thesis adds to the current literature by expanding on research demonstrating the significant effect of sex in two models of chronic pain. Furthermore, the results of this thesis outline how potential treatment for chronic pain may have different effects in males and females.

In Chapter 1 the role of sex in the formalin model of chronic inflammatory pain was explored. In this chapter, I demonstrate that while both male and female mice exhibit similar nociceptive behaviour in the formalin model, they respond differently to treatment with the monoamine oxidase inhibitor phenelzine and its metabolite PEH. Both drugs reduced nociception, but only in male mice. I found that pharmacological manipulation of the levels of serotonin, noradrenaline, and gamma-aminobutyric acid (GABA) revealed a serotonergic dependent nociceptive pathway in male mice and a more GABAergic dependent pathway in female mice.

In Chapters 2-4 I began to explore the benefits of a nonpharmacological intervention in Multiple Sclerosis (MS). Here again significant sex differences were found. Daily voluntary wheel running in the experimental autoimmune encephalomyelitis (EAE) model significantly improved disease scores in male but not female mice (Chapter 2). Improved disease scores in males with EAE who ran were associated with less axonal damage and oxidative stress at the chronic stage. Furthermore, voluntary wheel running also had effects in the brain in chronic EAE. I found that voluntary wheel running could modulate levels of neuroactive steroids associated with the EAE (Chapter 3). Female mice with EAE who ran had significantly increased levels of brain pregnenolone compared to male EAE mice who ran. In contrast, male mice with

EAE had significantly higher levels of brain allopregnanolone compared to female mice with EAE regardless of exercise. Allopregnanolone can be neuroprotective and therefore may account for the beneficial effects of running in male mice.

Finally, I explored the effect of voluntary wheel running and sex on nociceptive behavior in male and female mice with EAE. While chronic pain is a well-established secondary symptom of MS, few treatments or interventions exist to manage pain in the disease. I therefore wanted to explore whether voluntary wheel running could affect nociceptive behavior in male and female mice with EAE at the onset of disease. Interestingly, I found that daily voluntary wheel running reduced nociceptive behavior only in female mice with EAE (Chapter 4). I went on to show that this sex difference is due to different levels of pain-related circulating cytokines in the periphery and differences in dorsal root ganglia excitability between the sexes. Overall, my experiments demonstrate that male mice have a more pro-inflammatory profile when given access to a running wheel that ultimately contributes to their nociceptive behavior in the disease.

In summary, my work demonstrates the value of including sex as a variable in chronic pain research. In addition, it highlights how sex is an extremely important variable to consider when exploring both pharmacological and non-pharmacological interventions for chronic pain. Inclusion of variables such as sex in pre-clinical studies will ultimately lead to better translation of research and treatment for patients.

Preface

This thesis is an original work by Katherine A. Mifflin. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Assessing sensory function in EAE", AUP00000274, Approved Fall 2007-November 2018.

Parts of the introduction chapter have been published as K.A. Mifflin and B.J. Kerr. "Pain in autoimmune disorders." Journal of Neuroscience Research, vol. 95 issue 6,1282-1294. K.A. Mifflin, and B.J. Kerr. "The transition from acute to chronic pain: understanding how different biological systems interact." Canadian Journal of Anesthesia, vol. 62 issue 2, 112-122 and K.A. Mifflin and B.J. Kerr. "Sex- Related Differences in Acute and Chronic Pain: A Bench to Bedside Perspective." Canadian Journal of Anesthesia, vol. 60 issue 3, 221-226. For each of these reviews, I was responsible for concept formation, analysis, and manuscript composition. B.J. Kerr was the supervisory author and was involved with concept formation and manuscript and edits for all the listed reviews.

Chapter 1 of this thesis has been published as K.A. Mifflin, C. Benson, K. Thorburn, G.B. Baker, and B.J. Kerr. "Manipulation of neurotransmitter levels has differential effects on formalin evoked nociceptive behaviour in male and female mice." Journal of Pain, vol. 17 issue 4, 483-498. I was responsible for the concept formation, data collection and analysis, as well as the manuscript composition. C. Benson and K. Thorburn assisted with the data collection. G.B. Baker was involved with concept formation, and aided with data analysis and manuscript edits. B.J. Kerr was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 2 of this thesis has been published as K.A. Mifflin, E. Frieser, C. Benson, G.B. Baker, and B.J. Kerr. "Voluntary wheel running differentially affects disease outcomes in male and female mice with experimental autoimmune encephalomyelitis." Journal of Neuroimmunology, vol. 305, 135-144. I was responsible for the concept formation, data collection and analysis as well as the manuscript composition. E. Frieser assisted with the data collection. C Benson aided with data collection and concept formation. G.B. Baker was involved with concept formation, and manuscript edits. B.J. Kerr was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 3 of this thesis has been submitted to Neuroscience Letters as as K.A. Mifflin, E. G.B. Baker, and B.J. Kerr. "Effect of Voluntary Wheel Running on Neuroactive Steroid Levels in Murine Experimental Autoimmune Encephalomyelitis." I was responsible for the concept formation, running the animal experiments and tissue collection, data analysis as well as the manuscript composition. G Baker was involved with concept formation, data collection, and manuscript edits. B.J. Kerr was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 4 of this thesis is in preparation for and will be submitted to Journal of Neuroscience as K.A. Mifflin, M.S. Yousuf, K.C. Thorburn, J. Huang, M.E. Peréz-Muñoz, G. Tenorio, S. Dunn, J. Walter, K. Ballanyi, and B.J. Kerr. “Voluntary Wheel Running Reveals Sex Specific Nociceptive Factors in Murine Experimental Autoimmune Encephalomyelitis.” I was responsible for the concept formation, running the animal experiments and tissue collection and data analysis for figures 1-4. I was also responsible for manuscript composition. The DRG cultures, calcium imaging and data analysis in figures 5 and 6 were performed by M.S. Yousuf and K.C. Thorburn. M.S. Yousuf also aided in concept formation and editing of the final manuscript. J. Huang and G. Tenorio helped complete the western blots in figure 4. M.E. Peréz-Muñoz, S. Dunn, J. Walter, and K. Ballayni provided space and materials to complete the project as well as help with developing culture protocols. Finally, B.J. Kerr was the supervisory authors and involved in concept formation and manuscript composition.

When nothing in the lab seems to work, just remember

"Science my lads, is made up of mistakes, but they are mistakes which it is useful to make because they lead little by little to the truth"

-Jules Verne, A Journey to the Centre of the Earth

Acknowledgements

Completing my thesis has been a labour of love and is most definitely not something I accomplished on my own. I have many people to thank, but I must first thank my supervisor Dr. Bradley Kerr. Your support and guidance over these past six years has played a key role in the completion of this thesis. Your mentorship has allowed me to grow as a scientist and inspired me to pursue a career in science. I couldn't have asked for a better supervisor.

To my colleagues and summer students over the years: Kevin, Liam, Curtis, Gustavo, Saad, Ana, Timo, Kasia, Estzer, Ronja, and Jen, thank you all for your support throughout this entire process. Without our many coffee runs/chats, spontaneous scientific and statistical discussions, and hours running (and troubleshooting) experiments in the lab, I would not have been able to complete this thesis. You all made coming to the lab everyday an enjoyable experience; I could not have asked for better people to spend the last six years with.

I would also like to thank the members of my supervisory committee, Dr. Glen Baker and Dr. Peter Smith for their advice, guidance, and writing of many reference letters over the years. A special thank you to Dr. Baker and Gail for their immense knowledge of HPLC, without which the entire first chapter of this thesis would not exist. I would also like to thank members of the Walter lab, especially Maria-Elisa, and members of the Webber lab for helping me learn cell culture techniques.

Finally, I would like to thank my family, friends, and partner for all their love and willingness to listen to me complain after a long day. I am especially grateful to my parents, Randy and Daphane, for always supporting me in my educational pursuits and for believing unconditionally that I could accomplish anything I put my mind to. To my brother, Matthew, for always giving me a place to stay and for only occasionally rolling your eyes at how nerdy I am. To Kevin, for making me laugh until I cry and for always supporting me. Finally, to my friends both near and far, thank you for your encouragement, the late-night phone calls, the hours passed creating wonderful memories, and for always being willing to share a cup of coffee (or a beer).

I have been blessed to have so many wonderful and supportive people in my life, and nothing I have done would have been accomplished without them—thank you all so much.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

B2-AR: β2-adrenergic receptor
5-HT: 5-hydroxytryptamine
ALLO: allopregnanolone
AQP4: aquaporin 4
ANOVA: analysis of variance
BBB: blood-brain barrier
BDNF: brain derived neurotrophic factor
BH_4: tetahydrobiopterin
BSA: bovine serum albumin
CASPR2: contactin associated protein like 2
Cast: castrated
CD4: cluster of differentiation 4
CFA: Complete Freud's Adjuvant
CGRP: calcitonin gene related peptide
CM: conditioned media
CNS: central nervous system
CRPS: complex regional pain syndrome
CSF: cerebrospinal fluid
DHEA: dehydroepiandeosterone
DHT: dihydrotestosterone
DMSO: dimethyl sulfoxide
DRG: dorsal root ganglia
EAE: experimental autoimmune encephalomyelitis
GABA: γ-aminobutyric acid
GSH: glutathione
GSSG: glutathione disulfide
H_2O_2: hydrogen peroxide
HPLC: high performance liquid chromatography
HRP: horseradish peroxidase
Iba-1: ionized calcium-binding adapter molecule 1
IBC: N-iso-butyryl-L-cysteine
ISO: isopregnanolone
MAO: monoamine oxidase
MOG_{35-55}: myelin oligodendrocyte glycoprotein
MS: Multiple Sclerosis
N^2-AcPLZ: N^2-acetylphenelzine
NA: noradrenaline
NAS: neuroactive steroid
NMO: neuromyelitis optica
O.C.T.: optical cutting temperature
O_2: oxygen
O_2^-: superoxide
OPA: o-pthaldialdehyde
OVX: ovariectomized

PB: phosphate buffer
PBS: phosphate buffered saline
PEH: phenylethylidenehydrazine
PLZ: phenelzine
PML: progressive multifocal leukoencephalopathy
PNS: peripheral nervous system
PPAR: peroxisome proliferator-activated receptor
PPMS: primary progressive multiple sclerosis
PREG: pregnenolone
RCF: relative centrifugal field
RM ANOVA: repeated measures analysis of variance
ROS: reactive oxygen species
RNS: reactive nitrogen species
RRMS: relapsing remitting multiple sclerosis
SEM: standard error of the mean
SMI312/23: phosphorylated and non-phosphorylated neurofilament 200
SPMS: secondary progressive multiple sclerosis
SOD: superoxide dismutase
SSRI: selective serotonin reuptake inhibitor
TCA: tricyclic antidepressant
THDOC: tetrahydrodeoxycorticosterone
TNFα: tumor necrosis factor-α
VEH: vehicle
VGKCC: voltage gated potassium channel complex

Introduction

Pain is a universal sensation necessary for survival. From a purely sensory perspective, 'nociception', the neural process of encoding and processing noxious stimuli, has been observed from the simplest to the most complex organisms (Loeser and Treede, 2008; Moriarty et al., 2011). Although acute pain is an important warning signal for bodily harm, pain persisting long after tissue damage may become a clinical problem. Chronic pain can be defined as any pain lasting more than 3- 6 months (Moriarty et al., 2011). The World Health Organization has declared chronic pain to be one of the most common world health problems, with more than a quarter of the population suffering from chronic pain (Institute of Medicine (US) Committee on Advancing Pain Research, Care, 2011) . In Canada, estimates indicate that approximately 29% of the population suffers from chronic pain, with 80% of those individuals suffering from moderate to severe pain (Moulin et al., 2002). Despite the large prevalence of this condition, chronic pain is still poorly understood and notoriously difficult to treat. While acute pain is easily modeled in experimental settings as physiological or behavioural responses to noxious stimuli or tissue damage, chronic pain can arise from different types of tissue damage, may spontaneously arise without tissue damage, or can paradoxically exist in areas where damaged tissue is no longer present (i.e. phantom limb pain; Dworkin et al., 2007; Institute of Medicine (US) Committee on Advancing Pain Research, Care, 2011; Sherman, Sherman, & Parker, 1984). Chronic pain also violates the boundaries of the thresholds for what normally causes pain resulting in "*allodynia*" (pain to a stimuli that does not normally provoke pain), "*hyperalgesia*" (increased sensitivity to noxious stimulation), and "*sensitization*" (an increased responsiveness of nociceptive neurons to their normal input and/or recruitment of a response to normally subthreshold inputs; Loeser & Treede, 2008). Overall chronic pain is a paradoxical phenomenon with no adaptive purpose when compared to acute pain.

Models of Chronic Pain

While the mechanisms of chronic pain remain elusive, several animal models exist and have provided valuable insights into potential mechanisms. The models of chronic pain can be broken down roughly into three categories: inflammatory pain, neuropathic pain, and disease related pain. The section below will outline the major animal models in each of these categories.

Inflammatory Pain Models

Common models of inflammatory pain typically involve the injection of allogenic irritants that activate nociceptive fibers. These irritants can be injected into the skin, a joint, or visceral organs. The two most common irritants are capsaicin and carrageenan. Capsaicin is known to sensitize c-fibers through the activation of TRVP-1 channels and produces a reliable local inflammatory pain as measured by both thermal and mechanical hyperalgesia (see O'Neil et al., 2012 for a complete review of capsaicin). Importantly, capsaicin administration can also be used clinically, allowing for studies in clinical populations, as well as animal models (Simone et al., 1989). Additionally, this model led to the discovery of the TRPV-1 receptor, a receptor commonly involved in pain processing (O'Neill et al., 2012).

Carrageenan injection is similar to capsaicin as it also induces reliable inflammatory pain. However, carrageenan may be a better model of chronic inflammatory pain as enhanced nociceptive behaviours can been seen for up to 2 weeks after injection whereas capsaicin pain is more transient (Hargreaves et al., 1988; Radhakrishnan et al., 2003). Carrageenan inflammatory pain is thought to best model sprains and strains (Gregory et al., 2013) .

Formalin (a mixture of formaldehyde and water) is used to model tonic inflammatory pain. Formalin is injected into the hindpaw of a rodent and elicits a bi-phasic response seen by increases in nociceptive behaviour such as licking, lifting, and shaking (Dubuisson and Dennis, 1977). The first stage of the response is thought to be reflective of the initial injection, while the second phase is the response to central hyperexcitability of dorsal horn neurons (Martindale et al., 2001). While the formalin model is not reflective of any specific clinical condition, it is useful for modeling conditions in which there is moderately intense and long-lasting noxious stimulation.

Finally, Complete Freud's adjuvant (CFA) can also be injected into the tail, paw, muscle or joint to cause inflammation (Gregory et al., 2013). Injection of CFA produces a chronic inflammation associated with increased thermal and mechanical hyperalgesia at and outside of the injury site that persists for at least 7 days (Iadarola et al., 1988; Gregory et al., 2013). As a model of chronic inflammatory pain, CFA injections are thought to best model arthritis or tendonitis (Gregory et al., 2013).

Neuropathic Pain Models

Neuropathic pain is defined by the International Association for the Study of Pain as pain caused by a lesion or disease of the somatosensory nervous system. Damage can either be central or

peripheral. Neuropathic pain is often modeled in peripheral nerve injury modes, which have been essential in progressing our understanding of the mechanisms of chronic pain. These models are often done by inflicting damage to a peripheral nerve (Kumar et al., 2018). This can be done by direct injury to spinal nerves through ligation, transection, or strong chronic constriction of the nerve (Kumar et al., 2018). In the chronic constriction injury mode, the sciatic nerve is loosely ligated to disrupt but not completely obstruct blood flow, resulting in damage and inflammation. A partial nerve transection can also be used, where the sciatic nerve is lesioned to cause damage to a portion of the nerve. A spared nerve injury model is another model and is performed by tightly ligating either the peroneal or the tibial branch of the sciatic nerve (Bourquin et al., 2006). Though slightly different, all of these peripheral nerve injuries display reliable and sustained nociceptive behaviour as measured by decreased withdrawal thresholds to mechanical stimuli, increased thermal sensitivity, and guarding of the affected limbs (Gregory et al., 2013; Challa, 2015; Kumar et al., 2018). Furthermore, as the injury is often only performed on one side, the contralateral side can be used as a control or to examine the development of referred pain.

Overall, models of neuropathic pain have helped reveal several key mechanisms of chronic pain. These include the discoveries of wind-up and central sensitization, two processes essential in the development of chronic pain, the role of microglia in the development of chronic pain, and the role of purinergic receptors in these processes (Woolf, 1996; Li et al., 1999; Sorge et al., 2011, 2012; Tsuda, 2017). While neuropathic pain models have helped discover common mechanisms of chronic pain, the chronic pain associated with specific disease states may have unique or distinct mechanisms. Thus, the section below outlines chronic pain in different disease models.

Disease Related Models

Chronic pain is often a symptom of many diseases and contributes to a reduced quality of life for patients. There are several animal models of diseases that may be used to study chronic pain associated with the condition. Chronic pain often develops in autoimmune disease such as multiple sclerosis (MS) and rheumatoid arthritis, however as the development of chronic pain in autoimmune disease is discussed extensively below, these will not be discussed here.

There is a large body of research that has focused on pain in cancer. This is an especially active area of research as cancer pain can arise from certain types of cancer

themselves or from treatment with chemotherapy. Cancer pain is often a mix of inflammatory, neuropathic and ischemic mechanisms of pain. Models of cancer and cancer pain often involve the injection of tumor cells into various tissues. In bone cancer, tumors are injected into bone, while in melanoma models, melanoma cells are often injected into the plantar pad of the paw (Portenoy and Ahmed, 2018). Both of these models demonstrate enhanced nociceptive behaviour and are associated with changes in inflammation in the spinal cord environment and dorsal root ganglia (DRG) (Portenoy and Ahmed, 2018). Enhanced nociceptive behaviours have also been seen in models of head and neck cancer, as well as peritoneal cancer (Portenoy and Ahmed, 2018). Additionally, several chemotherapies can lead to in peripheral neuropathy. This can be modeled in rodents by using common chemotherapeutics such as vincristine, paclitaxel, oxaliplatin or bortezomib (Hama and Takamatsu, 2016). All of these chemotherapeutics induce mechanical allodynia, mechanical hyperalgesia, and thermal hypersensitivity (Hama and Takamatsu, 2016). These models are useful for better understanding the mechanism of chronic pain in cancer and how best to modify treatments to reduce chemotherapy induced pain.

Spinal cord injury is an injury/disease state associated with chronic pain. There are several models of spinal cord injury, which are defined by how the damage is inflicted to the spinal columns. Injury can be inflicted by either partial or full transection, crush, contusion, ischemia, or excitotoxicty (Gregory et al., 2013; Kumar et al., 2018). All of these models are associated with the development of sustained hypersensitivity. This is demonstrated by development of mechanical and thermal hyperalgesia (Gregory et al., 2013; Siddall and Middleton, 2015). Furthermore, the development of chronic pain could also be a concern in many of the treatments being developed for spinal cord injury. As methods are devised to try and re-establish lost connections in the injured spinal cord it is important to assess whether the connections being made promote enhanced nociception through increased sprouting of serotonergic or noradrenergic neurons.

Variables that Influence Chronic Pain

Sex

Sex differences have emerged over the past several years as one of the major factors that influence chronic pain. Research in both clinical populations and animal models show males and

females experience painful stimuli differently. The biological mechanisms leading to the development of chronic pain are also vastly different between the two sexes.

Studies exploring the sensitivity to acute pain stimuli have shown that, in general, females are more sensitive to noxious stimulation (Riley et al., 1998). This general sex difference carries over to studies of the prevalence of chronic pain conditions, where chronic pain conditions are more commonly seen in females (Moulin et al., 2002). Given these strong clinical differences, a large body of research has focused on understanding the underlying biological mechanisms of these sex differences. Overall, this research suggests that males and females have different biological responses to chronic pain. For example, a recent study in this area found male and female mice use different immune cell mediated pathways in the development of neuropathic pain after spared nerve injury (Sorge et al., 2015). This study found that the development of mechanical hypersensitivity in male mice was microglia dependent. Female mice, however, were microglia independent and the pain was mediated by T-lymphocytes (Sorge et al., 2015). These sex differences were dependent on testosterone levels (Sorge et al., 2015). Other studies have also demonstrated sex specific pathways. For example, a study examining the effectiveness of morphine between the sexes found that opioid receptors responsible for morphine analgesia are upregulated in females due to increased levels of estrogen over the estrus cycle (Chakrabarti et al., 2010). Due to this regulation of opioid receptors by estrogen, morphine analgesia appears to be mediated by the κ- opioid receptor gene in females and not males (Chakrabarti et al., 2010). Additional studies show that morphine analgesia is dependent on N-methyl-D-asparate-type glutamate receptors in males, while females are dependent on the melanocortin 1 receptor (Mogil et al., 2003; Juni et al., 2010). Finally, major sex differences have also been seen in a well-established pathway of chronic pain development: toll-like receptor 4 (TLR-4) activation on microglia. Studies exploring sex in this chronic pain mechanism have found in the C3H/HeJ mouse strain with a mutation in the TLR4 gene, that only male mice show reduced pain sensitivity (Tanga et al., 2005). Furthermore, when female mice are given either agonists or antagonists to manipulate this receptor, no difference in nociceptive behaviour is seen (Bettoni et al., 2008). This study highlights a sex specific chronic pain pathway.

Age

Chronic pain is seen in individuals of all ages, however little research has been done to explore how the experience or mechanisms of chronic pain may differ between age groups. Studies in other areas of research have found that disease biology often changes with age, indicating that age may be an important variable to consider. Importantly, studies exploring the prevalence rates of chronic pain conditions across different age groups show that chronic pain etiology changes with age. It is also important to note that while pediatric chronic pain conditions do occur, their prevalence rates are lower than those of adults. Furthermore, a recent study exploring pain in the elderly (ages 70-90) found that there are significant age differences in rates of visceral, headache, and peripheral pain within this small age range (Rottenberg et al., 2015). For example, rates of visceral pain decreased after 85 years of age to an almost undetectable level in the population (Rottenberg et al., 2015). Interestingly, this reduction in pain with age was seen in measures of headache pain as well (Rottenberg et al., 2015). While the rates of peripheral pain, such as chronic lower back pain or joint pain, also decreased with age there was still a higher prevalence (14-25%) rate of peripheral pain compared to other types of pain at age 90 (Rottenberg et al., 2015). Other studies examining migraine pain have also found that the number of migraines decreases with age. A reduction of pain conditions in the elderly population is a bit surprising, as previous reports of changes with prevalence with age over time report increases. However, these studies typically report on chronic pain in young adult to middle age (45-65 years of age). Interestingly, when these studies are extended past 65, a plateau in prevalence rates can be seen (Johannes et al., 2010). This was also seen in a study of lower back pain, where chronic lower back pain peaked at ages 51-60 but then decreased in the elderly population studied (Hüllemann et al., 2018). This suggests that there is a difference in the mechanisms behind the development of chronic pain between middle aged and elderly individuals.

Interestingly, there was a difference in the type of pain experience with age. Neuropathic pain (pressure pain/pain attacks) decreases in the elderly, but measures of nociceptive pain (burning/prickling) are increased, suggesting possible differences in pathology (Hüllemann et al., 2018). Research using animal models also suggests that the pathogenesis of pain is different between young and aged mice. For example, a recent study of neuropathic pain in young and aged mice with spared nerve ligation found that levels of brain derived neurotrophic factor (BDNF) in the hippocampus were decreased in aged mice compared to

young mice (Tateiwa et al., 2018). Furthermore, this decrease in hippocampal BDNF was associated with increased hyperalgesia in aged mice (Tateiwa et al., 2018).

Other animal model studies of specific chronic pain conditions reflect the population studies showing that chronic pain decreases or plateaus with advanced ages. A study examining calcitonin-gene related peptide (CGRP) in head and neck pain found that there was a decrease in CGRP in the superior cervical ganglion in aged mice (Mitsuoka et al., 2018). This reduction in CGRP levels with age is thought to be related to the decrease in migraine seen with age (Mitsuoka et al., 2018). A decrease in pain with age is also seen in a model of arthritic pain, where mice at 3, 15, and 22 months of age had decreasing nociceptive behaviours with age despite having similar levels of injury (Ogbonna et al., 2015). These mice also had reduced spinal microglia activation (Ogbonna et al., 2015). This study also found that there was decreased nociceptive behaviour with age in the zymozan model of inflammation (Ogbonna et al., 2015). Overall these studies in both clinical populations and animal models demonstrate how chronic pain can change with age. This is also an important variable to consider when trying to translate research, as the results may change depending on the age of the population of interest. In fact, it has been suggested that one of the reasons for the failure of bench to bedside translation for chronic pain therapeutic development is the lack of studies considering age as a variable (Yezierski and Hansson, 2018).

Genetics

Twin studies have indicated that there appears to be a genetic connection in the development of chronic pain. A study exploring the proportion of risk of developing a chronic pain condition driven by genetic background found that the heritability of chronic pain conditions was 25-50% (Nielsen et al., 2012). The highest rate of heritability was seen in chronic widespread pain and migraine conditions (Nielsen et al., 2012). A separate twin study found that the genetic contribution accounted for about 50% of the likelihood of developing fibromyalgia (Kato et al., 2006). In addition to these studies, studies of rare genetic mutations in families have helped reveal the role of several ion channels in pain processing. Interestingly one of the first studies of this type examined pain-free individuals instead of those with a chronic pain condition (Cox et al., 2006). It was found that a lack of the the voltage gated-sodium channel $Na_v1.7$ in peripheral neurons resulted in hypoalgesia (Cox et al., 2006). While these studies are beneficial in

discovering channels involved in normal pain processing, given the rarity of these mutations, they do not give much insight into common chronic pain conditions. Due to this, many researchers have taken a wider approach to determine more common genetic variations associated with the development of chronic pain. A study exploring common single nucleotide polymorphisms (SNPs) associated with chronic pain conditions found 846 SNPs associated with various chronic pain conditions (Sexton et al., 2018). One of the most commonly affected genes in chronic pain is COMT, the gene encoding for the catechol-O-methyltransferase enzyme, which is responsible for degradation of the catecholamine neurotransmitters dopamine, adrenaline, and noradrenaline (Diatchenko et al., 2005). This gene's activity is often reduced in chronic pain, which results in an increase in pain sensitivity (Diatchenko et al., 2005). This is thought to be due to either lack of breakdown of noradrenaline or an associated reduction in enkephalins in the CNS (Diatchenko et al., 2005). A splice variant of COMT called (a)-COMT can also contribute to pain, as this variant leads to increased breakdown of neurotransmitters such as dopamine (Sexton et al., 2018). Changes in the COMT gene have been seen in fibromyalgia, postoperative pain, migraine, and low back pain (Sexton et al., 2018).

Another common gene disrupted in chronic pain conditions is the gene GCH1, which is the rate-liming enzyme for tetahydrobiopterin (BH_4) synthesis (Tegeder et al., 2006). BH_4 is also involved in catecholamine production and a cofactor in nitric oxide and serotonin biosynthesis. It has been found that increased activity of GCH1 and the subsequent increase in BH_4 synthesis in peripheral neurons is related to peripheral neuropathic and inflammatory pain (Tegeder et al., 2006). This is thought to be due to increased catecholamine production via overactivity of GCH1. Interestingly, both dysregulation of COMT and GCH1/BH_4 are involved in neurotransmitter changes associated with chronic pain development. Disruptions in neurotransmitters in chronic pain have long been studied and are discussed below.

Neurotransmitters in Pain

Changes in neurotransmitters throughout the CNS have long been linked to the development of chronic pain. In fact, many of the current treatments for chronic pain (discussed below) work to increase the levels of specific neurotransmitters.

Noradrenaline

Increasing the levels of NA can alleviate many different types of pain, though how exactly NA acts to affect pain is still unclear (Dworkin et al., 2007; Attal et al., 2010; Saarto and Wiffen, 2010; Bohren et al., 2013). Increased levels of NA in the central nervous system potentiate descending inhibitory pathways in the spinal cord (Xu et al., 1999; Pertovaara, 2006; Hughes et al., 2013a). In addition, recent work has shown that NA and opioids work synergistically to counter pain. This can be seen with drugs like tapentadol, which is an agonist of the mu-opioid receptor and a noradrenaline reuptake inhibitor (Meske et al., 2014). Other antidepressants dampen neuropathic pain in an indirect manner by recruiting NA from sympathetic fibers sprouting in the DRG. This then stimulates local β2-adrenergic receptors on non-neuronal cells to decrease membrane bound TNFα production, the reduction of which is anti-allodynic (Bohren et al., 2013).

Serotonin

While in general, increased levels of NA promote analgesia, the role of 5-HT in pain is much more complex. Research done on the role of 5-HT in pain indicates that 5-HT acts differently in the central and peripheral nervous systems (PNS). 5-HT released from the nucleus raphe magnus in the brainstem causes an excitation of inhibitory interneurons in the spinal cord to reduce hypersensitivity (Sommer, 2004a). Thus, the loss of 5-HT regulated descending inhibitory pathways can lead to the development of neuropathic pain. Decreases in spinal cord 5-HT following nerve injury also decrease inhibitory pathway activation and lead to the development of central sensitization and chronic pain (Sommer, 2004a; Liu et al., 2005). Intrathecal injections of 5-HT after spinal cord injury also reverse the associated depletion of 5-HT and counteract the development of mechanical allodynia (Hains et al., 2002). In addition, one study has shown that a reduction in the levels of 5- HT in the nucleus raphe magnus is associated with a lack of opioid analgesia in both sciatic nerve ligation and diabetic induced neuropathy (Sounvoravong et al., 2004).

Interestingly, 5-HT appears to act differently in the PNS, especially in inflammatory pain. Multiple studies have shown that there is a release of 5-HT in both the central and peripheral nervous system during inflammation, which may contribute to the development of pain (Zhang et al., 2000; Hains et al., 2002; Palazzo et al., 2004; Sommer, 2004a). 5-HT released from circulating mast cells and platelets during inflammation appears to act as a powerful pro-

inflammatory agent and can result in hyperalgesia (Wu et al., 2001; Sommer, 2004a; Ferjan and Lipnik-Stangelj, 2013). This is thought to be related to changes in 5-HT receptor expression in the DRG in response to inflammation (Wu et al., 2001; Liu et al., 2005). A study also found that TCAs reduce mast cell release of 5-HT in the periphery, which may account for some of the analgesic effects of TCAs (Ferjan and Lipnik-Stangelj, 2013). Overall, the role of 5-HT in pain is complex and still not fully understood. Current research regarding 5-HT and pain suggests that a balance is needed between the inhibitory and excitatory actions of 5-HT to control/prevent the development of chronic pain.

Gamma-Aminobutyric Acid

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter, is also involved in pain. Similar to the reductions seen in NA and 5-HT, loss of spinal cord GABA has been linked to the development of chronic pain (Moore et al., 2002; Zeilhofer et al., 2009). Changes in GABA-mediated neuronal excitability also contribute to the development of central sensitization and chronic pain. After nerve injury, there is a reduction in the potassium-chloride transporter, KCC2, and an increase in microglial derived brain derived neurotrophic factor (BDNF) release in the spinal cord (Coull et al., 2003, 2005). The loss of KCC2 leads to an increase in neuronal intracellular chloride levels, resulting in $GABA_A$-receptor/GlyR-receptor mediated depolarization and increased neuronal excitability (Coull et al., 2003, 2005). A similar pattern of events has also been seen in the nucleus raphe magnus of the brainstem, a supraspinal site for pain modulation, showing that altered GABAergic inhibition in the brainstem can activate descending pain-facilitating pathways to cause chronic pain (Zhang et al., 2013).

Manipulating GABA in the spinal cord and brain has been shown to alter hyperalgesia. For example, increasing GABA in the rostral agranular insular cortex, an area of the cortex involved in pain perception, produced lasting analgesia by increasing descending inhibition of spinal nociceptors (Jasmin et al., 2003). It is also interesting to note that when $GABA_B$ receptors were selectively targeted in the insular cortex, there was an increase in hyperalgesia, suggesting that GABA acts in multiple pathways to control pain perception and a balance is needed to control pain (Jasmin et al., 2003). In addition, drugs that act at $GABA_A$ receptors, such as benzodiazepines, are anti-hyperalgesic (i.e. prevents the development of

hyperalgesia) as they help reverse the loss of GABAergic tone in chronic pain (Knabl et al., 2008, 2009).

Current Therapies for Chronic Pain

Chronic pain conditions, and especially neuropathic pain conditions, are notoriously difficult to treat. As a result, there are several categories of pharmacological therapies used to manage chronic pain. In addition, more complementary and/or integrative approaches to chronic pain management have arisen, as one drug treatment often fails to manage chronic pain.

Antidepressants and Anticonvulsants

As outlined above, changes in neurotransmitter levels are a key contributor to the development of chronic pain. It should therefore be no surprise that antidepressants are considered the first line treatment for chronic pain, and especially for neuropathic pain. Antidepressants are also often prescribed for chronic pain conditions given the frequent instances of comorbidity between chronic pain and depression (Fornasari, 2017). It is important to note however, that relief from chronic pain often comes from a lower dose than that needed for treatment of depression. Given that multiple neurotransmitter systems are disrupted in chronic pain, "dirty" antidepressants, such as the TCAs desipramine and amitriptyline, are more effective than more selective antidepressants (Dworkin et al., 2007; Finnerup et al., 2015). TCAs can directly interfere with the development of central sensitization by blocking NMDA receptors in the spinal cord and by increasing available levels of neurotransmitters at the synapse (Kremer et al., 2016). Similarly, antidepressants which act on both serotonin and noradrenaline (serotonin and noradrenaline reuptake inhibitors, SNRI) are also effective in many chronic pain conditions such as painful polyneuropathic, post-herpetic neuralgia, lower back pain, and diabetic neuropathy (Finnerup et al., 2015; Fornasari, 2017).

Anticonvulsants are also effective for the management of chronic pain and are also considered first line treatment of many chronic pain conditions. The two most commonly prescribed anticonvulsants are pregabalin and gabapentin, both gabapentinoids (Fornasari, 2017). Gabapentinoids have been shown to have beneficial effects in post-herpetic neuralgia, lower back pain, diabetic neuropathy, and polyneuropathy (Fornasari, 2017). Interestingly, while both of these drugs were originally derived from GABA they do not actually act on the GABAergic

system. Instead, both drugs work by binding to subunits of voltage gated calcium channels in the CNS (Stahl et al., 2013). Levels of voltage gated calcium channels are increased in chronic pain states and contribute to the sustainment and development of chronic pain by maintaining aberrant neurotransmission in the spinal cord (Stahl et al., 2013; Fornasari, 2017). The gabapentinoids therefore likely influence the number of available voltage gated calcium channels in the plasma membrane. There is also some evidence to suggest that these drugs alter descending noradrenergic and serotonergic activity to modulate nociception in the spinal cord, making them powerful analgesics in the context of chronic pain (Bockbrader et al., 2010).

While both antidepressants and anticonvulsants are useful for the management of chronic pain, they are often associated with many side effects. For example, TCAs are associated with the development of anticholinergic effects such as dry mouth, orthostatic hypotension, urinary retention and constipation (Fornasari, 2017). The most common side effect of SNRIs is nausea (Fornasari, 2017). Thus, while these drugs may be effective, they are not tolerated well by patients. Anticonvulsants on the other hand seem to have fewer side effects, with dizziness and somnolence being most commonly reported. These can often be minimized however, by dose adjustment (Bockbrader et al., 2010). Thus, both antidepressants and anticonvulsant therapies are effective and well tolerated in chronic pain populations. However, it is important to note that many types of chronic pain are still intractable. This means that more research is needed to develop new drugs or to explore new approaches to pain management, such as those discussed below.

Opioids

Opioids (e.g. morphine, oxycodone, hydromorphine, tramadol) are extremely effective analgesics and have been used for hundreds of years to manage both acute and chronic pain. Opioids exert their actions through the opioid receptor family (mu, kappa, and delta) in the brain, brainstem, spinal cord, and PNS. Most of the analgesic effect of opioids is mediated through mu receptors in the dorsal horn of the spinal cord at the terminal of afferent nociceptors or on the dendrites of second order neurons and interneurons (Fornasari, 2017). The activation of the mu opioid receptor causes the inhibition of calcium ion channels and activation of potassium ion channels to dampen neuronal activity by blocking synaptic transmission (Fornasari, 2017). While extremely effective for acute pain management, there are several issues with opioid use for

chronic pain conditions. The major concern for the use of opioids is the rapid development of tolerance, low tolerability by patients, and high risk of addiction (Trang et al., 2015). In fact, it is thought that the over prescribing of opioids for the management of pain has helped contribute to the current opioid crisis (Compton & Volkow, 2006; Okie, 2010; Trang et al., 2015). In addition to these issues, the overuse of opioids can lead to the development of a condition called morphine hyperalgesia. This is when overuse of opioids to manage chronic pain actually leads to the development of paradoxical chronic pain. The development of mechanical hyperalgesia is thought to be due to the activation of a mu dependent expression of P2X4 receptors on microglia in the spinal cord that disrupts chloride homeostasis, leading to pain hypersensitivity (Ferrini et al., 2013). Thus, while opioids can be used effectively to treat pain for brief periods, their prolonged use in chronic pain conditions is contraindicated.

Alternative Approaches to Pain Management

Given the adverse effects and issues associated with many drugs used to treat chronic pain, new research has begun to explore non-pharmacological interventions. This has led to the development of multidisciplinary teams of physicians, physiotherapists, psychologists, anesthesiologists, and nurses working together to help treat chronic pain. Meta-analyses and reviews have explored the effectiveness of alternative therapies such as exercise, diet, yoga, relaxation, tai chi, massage, cognitive behavioural therapy, and acupuncture in chronic pain (Crawford et al., 2014; Geneen et al., 2017; Lin et al., 2017; Aman et al., 2018) . So far there seems to be strong evidence for the use of several of these interventions. Two different meta-analyses exploring yoga and tai chi in chronic pain demonstrated moderate effects on chronic pain (Crawford et al., 2014; Lin et al., 2017). Furthermore, exploration of the effectiveness of exercise in chronic pain has revealed that exercise can moderately reduce chronic pain in general (Geneen et al., 2017). Stronger evidence for the pain reducing effects of exercise have been seen in more specific chronic pain conditions, such as lower back pain (Searle et al., 2015). The difference between the effects of exercise for chronic pain in general compared to more specific conditions are likely due to the lack of standardization between exercise interventions in the different studies. In contrast, there is relatively strong evidence for the use of cognitive behavioural therapy (Probyn et al., 2017; Schütze et al., 2018). This is an intervention that focuses on reducing the catastrophizing behaviour seen in many chronic pain patients, and

teaching patients to set attainable goals for pain management (Aman et al., 2018). Other interventions, such as acupuncture, have mixed results, with some analyses reporting beneficial effects and others reporting little evidence of a beneficial effect on pain management (Crawford et al., 2014; Lin et al., 2017; Aman et al., 2018). While the evidence to date for alternative interventions for the management of chronic pain only demonstrates moderate to mixed effectiveness, they do provide an new area for research for the management of chronic pain outside of current therapies such as antidepressants and anti-convulsants.

Pain in Autoimmune Diseases

Although often considered rare, there are over 80 known autoimmune diseases according to the Progress in Autoimmune Disease Research report by the National Institutes of Health's Autoimmune Diseases Coordinating Committee (ADCC, 2005). Each of these diseases has a unique autoantibody profile and primary clinical manifestations. Some examples of the more recognizable autoimmune disorders include MS, rheumatoid arthritis, type 1 diabetes, psoriasis, and irritable bowel syndrome. Although each disease varies in its presentation, the common underlying thread is the presence of autoantibodies and autoreactive T cells, which cause harm to the patient. These are thought to attack the various tissues and/or organs targeted in the individual disease. For example, autoantibodies against components of the myelin sheath cause the damage and clinical symptoms associated with MS. The main cause of most autoimmune diseases is not yet known, although many studies have linked these diseases to both genetic/epigenetic and environmental factors (Miller et al., 2012; Selmi et al., 2012; Aslani et al., 2016). Given the chronic nature of most autoimmune diseases, they have a significant impact on the health-care system and the quality of life of patients. This makes understanding their underlying biology and treatment imperative.

Although there is a large variety of autoimmune disorders with different symptomologies, pain appears to be a common feature of most of these conditions. A quick search of the autoimmune diseases listed by the National Institute of Health's National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) and pain symptoms reveals that a shocking number of autoimmune diseases are associated with pain. In the list provided by NIAMS, 19 of 24 of the autoimmune diseases listed (79%) had research linking the disease to pain (Table I). In addition, other autoimmune diseases not listed by the NIAMS, such as Crohn's

and celiac diseases, have been linked to pain in the peripheral nervous system (Morrison et al., 2013; Green et al., 2015). Multiple studies have also linked increased bodily pain scores in autoimmune diseases with reduced quality of life for patients.

Given that many autoimmune diseases are associated with painful symptoms, it is not surprising that chronic pain has been implicated as a comorbid disorder in several autoimmune diseases. These include neuropathic pain in MS, painful diabetic neuropathy in diabetes, and chronic abdominal pain in Crohn's disease and Sjogren's syndrome (Pöllmann and Feneberg, 2008; Morrison et al., 2013; Downs and Faulkner, 2015; Grossman and Tagliavini, 2015). Given that pain severely affects quality of life and disease burden, it is important to understand the link between autoimmune diseases and the development of chronic pain. It is also important to acknowledge that the pain experienced in each autoimmune disease is unique. This is not surprising given the wide range of pathologies and symptoms of autoimmune diseases. For example, pain in MS is often neuropathic or mixed in nature and occurs in approximately 50% of patients (Österberg et al., 2005; O'Connor et al., 2008). Neuropathic pain is also common in diabetes, but often in the form of peripheral neuropathies, with approximately 50% of people experiencing distal sensorimortor polyneuropathies and autonomic neuropathies (Boulton et al., 2005; Downs and Faulkner, 2015). Pain associated with inflammatory bowel disease is usually abdominal pain (90% of patients) and chronic in approximately 38% of patients (Morrison et al., 2013). Pain in inflammatory bowel disease was also found to be associated with reduced quality of life, higher disease activity levels, and more depression and anxiety (Morrison et al., 2013). Sjogren's syndrome, however, is associated with diffuse pain and fibromyalgia along with dryness-related discomfort and severe fatigue that are associated with reduced quality of life (Jousse-Joulin et al., 2013; Grossman and Tagliavini, 2015; Milin et al., 2016). Given the variety of autoimmune diseases and their specific pain symptoms/classifications, it is unlikely that the specific underlying biological causes will be the same in each disease, but common mechanisms may exist. The sections below examine a possible common underlying biological link between chronic pain and autoimmunity by exploring the role of the immune system and autoantibodies.

Involvement of the Immune System in Autoimmune Disease and Chronic Pain Development

It goes without saying that a dysregulation of the immune system is inextricably linked to autoimmune disease, but the development of chronic pain is also closely related to aberrant activation of the immune system. It is therefore not surprising that the immune system has been implicated in the development of chronic pain associated with autoimmune diseases. A large body of research has explored the various and intricate connections between the development of chronic pain and the immune system. In general, it is thought that initial injury in the peripheral system triggers an acute inflammatory response, which through the release of various inflammatory factors (known as the "inflammatory soup") contributes to immune cell recruitment and peripheral sensitization (Scholz and Woolf, 2007). This then contributes to the infiltration of immune cells such as macrophages and T lymphocytes into the DRG, and Schwann cells begin to proliferate and dedifferentiate (Scholz and Woolf, 2007). This leads to the sensitization of neuronal cell bodies, increased excitability, and hypersensitization.
In response to this, microglia and astrocytes may be activated in the dorsal horn of the spinal cord, where various proinflammatory factors are released, leading to the sensitization of spinal circuits and chronic pain (see (Calvo, Dawes, & Bennett, 2012; Mifflin & Kerr, 2014; Ren & Dubner, 2010; Scholz & Woolf, 2007; Verma, Sheikh, & Ahmed, 2015) for review). However, much of this work has used models of neuropathic pain that involve overt trauma to a peripheral nerve and therefore might not directly be modeling the processes involved in autoimmune diseases. Although some of these pathways may be involved in autoimmune disease-associated chronic pain, most of the work exploring the immune system, chronic pain, and autoimmune disease has focused on the involvement of T cells and microglia. Therefore, I will briefly outline the involvement of T cells and microglia in chronic pain and autoimmune diseases before exploring a new area of study: autoantibodies and pain.

Autoimmune Diseases, T Cells, and Chronic Pain

The involvement of T cells in various autoimmune disorders has been well established. Both $CD4^+$ and $CD8^+$ T-cell subtypes have been implicated in several autoimmune diseases, such as MS, type 1 diabetes, and Guillain-Barre syndrome (Salou et al., 2015; Yang et al., 2015; Ferretti and La Cava, 2016). An imbalance in T-helper 1 (Th1)/Th2 immunity has traditionally been thought to contribute to autoimmune disease development (O'Shea et al., 2002; Coffman, 2006;

Moudgil, 2015). The Th1 subset of T cells is thought to be pro-inflammatory and is linked to the release of pro-inflammatory cytokines such as interleukin (IL)-1b, IL-6, tumor necrosis factor-α (TNFα), and IL-12, whereas the Th2 subset is thought to be more anti-inflammatory and related to the release of anti-inflammatory cytokines such as IL-10, IL-4, IL-11, and IL-13 (Romagnani, 2000). Normally, there is a balance between the two subsets to keep inflammation mediated by pro-inflammatory cytokines and pathogenic T cells in balance with anti-inflammatory cytokines and regulatory T cells (Coffman, 2006; Moudgil, 2015). For example, the progression and initial disease stages of MS have been linked to Th1/Th2 imbalances. This is based on evidence from the experimental autoimmune encephalomyelitis animal model, which is primarily a $CD4^{+}$/ Th1-mediated disease (Begolka et al., 1998; Sospedra and Martin, 2005). However, research over the past decade has shown that this dichotic view of T-cell immunity is too simplistic and that other players are involved (O'Shea et al., 2002). For example, it has come to light that the Th17 cell population is often involved in the development of autoimmune diseases (Annunziato et al., 2009; Hueber et al., 2010; Steinman, 2010; Leonardi et al., 2012; Gisondi et al., 2014). Th17 cells have been found to play a central role in both inflammation and autoimmunity by producing pro-inflammatory cytokines such as IL-17, IL-17F, and IL-22 (Moynes et al., 2014). These factors activate microglia and can directly act on neurons, promoting immune cell infiltration (for a complete review see Moynes et al., 2014). Overall, Th17 cells have been implicated in rheumatoid arthritis, uveitis, MS, Crohn's disease, psoriatic arthritis, and psoriasis (Annunziato et al., 2009; Hueber et al., 2010; Steinman, 2010; Leonardi et al., 2012; Gisondi et al., 2014). Antibody therapies against IL-17 have been shown to be beneficial in some autoimmune diseases such as MS, rheumatoid arthritis, and plaque psoriasis (Hueber et al., 2010; Steinman, 2010; Leonardi et al., 2012).

Various T-cell populations have also been linked to the development of chronic pain, such as the Th1/Th2 system and Th17 (Austin and Moalem-Taylor, 2010). For example, T cells were differentiated from a model of peripheral nerve injury into two polarized populations based on Th1 and Th2 cytokine profiles. Passive transfer of the Th1 subset into nude rats enhanced pain hypersensitivity, whereas passive transfer of Th2 subset into heterozygous rats attenuated pain hypersensitivity, suggesting a role for T cells in the development of pain (Moalem et al., 2004). The influx of T cells into the CNS has also been linked to thermal hyperalgesia, mechanical allodynia, and cold allodynia in mice (Aicher, Silverman, Winkler, & Bebo, 2004b;

Grace, Rolan, & Hutchinson, 2011; Olechowski, Truong, & Kerr, 2009). This was seen in mice with experimental autoimmune encephalomyelitis and experimental autoimmune neuritis (an inflammatory demyelinating disease in the PNS), suggesting a role for T-cell-mediated pain in autoimmune disease (Aicher et al., 2004a; Olechowski et al., 2009). T cells also mediate the maintenance of chronic pain through proinflammatory interactions with glial cells in the spinal cord, contributing to central sensitization (Grace et al., 2011). Chronic pain associated with autoimmune diseases is therefore often linked to T-cell infiltration. The Th17 population is also thought to contribute to the development of chronic pain through the production of IL-1(Li et al., 2013; Day et al., 2014; Moynes et al., 2014). For example, the release of IL-17 from Th17 cells onto keratinocytes in plaque psoriasis associated pain increases chemokine expression, recruiting additional immune cells to lesion sites and worsening the disease (Girolomoni et al., 2012). In fact, ixekizumab, an anti- IL-17 monoclonal antibody treatment, was shown to reduce both disease severity and the associated pain in patients with psoriasis (Leonardi et al., 2012). Furthermore, an animal model of Guillain-Barré syndrome called experimental autoimmune neuritis has been linked to T-cell infiltration and an increase in antigen-presenting cells in the sciatic nerve that correlates with pain behaviors in the model (Moalem-Taylor et al., 2007). Another model of a spontaneous autoimmune peripheral polyneuropathy shows that, despite the model being in a $CD4^{-/-}$ mouse, there was a massive infiltration of $CD8^{+}$ T cells in peripheral nerves (Yang et al., 2014, 2015). The $CD8^{+}$ T-cell infiltration was also associated with enhanced nociceptive behaviors in the model (Yang et al., 2014, 2015). Both of these studies strongly implicate a role for T cells in pain associated with Guillain-Barré syndrome. Further studies have also linked autoimmune diseases, pain, and T-cell infiltration. For example, one study showed that increases in $CD4^{+}$ and $CD8^{1+}$T cells through a possible chemokine (CCL2)-mediated pathway in a mouse model of chronic prostatitis are associated with increased nociceptive behavior in the model (Quick et al., 2012). An additional study examining chronic prostatitis also found an increase in the number of $CD4^{+/}$IL-17A T cells from mouse lymph tissue and patient urinary samples (Murphy et al., 2015). IL-17 released from these cells was responsible for the development of pelvic pain in an animal model and also in patients with the disease (Murphy et al., 2015). Finally, both Th1 and Th17 T cell populations have been implicated in arthritis (Sarkar et al., 2010; Ebbinghaus et al., 2012). Furthermore, a reduction in Th1 and Th17 T cell populations through a sympathectomy has been associated with an attenuation of inflammation,

pro-inflammatory immune parameters, and hyperalgesia in an animal model of arthritis (Ebbinghaus et al., 2012).

Overall, these studies demonstrate a strong connection between autoimmune disease-related pain and T-cell infiltration. This is no surprise given that most autoimmune diseases are thought to be T cell mediated. These insights into the connection between the immune system and pain do, however, provide insight into T-cell-related targets that might help not only in alleviating disease pathology but also in disease-associated pain.

Microglia, Autoimmune Disease, and the Development of Chronic Pain

Microglia are considered the inflammatory cells of the CNS and make up approximately 10% of the total adult cells there (Salter and Beggs, 2014). Microglia are thought to survey the environment constantly with their processes to ensure a healthy state in the CNS (Salter and Beggs, 2014). Recent studies have also implicated microglia in the development of chronic pain. Several studies have shown that, after injury, microglia become activated and are recruited to the site of injury through various pathways. One such pathway involves the release of fractalkine (CXC3CL1), a neuronal chemokine, and its microglial receptor CX3CR1 (Clark et al., 2009; Hathway et al., 2009). The release of fractalkine after injury increases microglial activation in the spinal dorsal horn and then contributes to pain sensitivity (Clark et al., 2009; Hathway et al., 2009). Activation of spinal microglia can also cause the release of pro-inflammatory cytokines such as IL-1b that contribute to pain hypersensitivity (Calvo et al., 2012). One of the more recently described pathways linking microglia activation to pain is through purinergic receptors and ATP. The release of ATP after injury activates P2X4 receptors on microglia and causes the release of brain-derived neurotrophic factor (BDNF) from microglia (Trang et al., 2009; Beggs et al., 2012). Secreted BDNF can then bind to TrkB receptors on nociceptive neurons in the spinal dorsal horn. The binding of BDNF can trigger downregulation of the potassium chloride cotransporter KCC2, resulting in a shift in the chloride anion gradient. As a consequence of this shift, the subsequent binding of GABA to the $GABA_A$ receptor, which is normally inhibitory, now leads to the disinhibition of inhibitory neurons (Coull et al., 2005). This is thought to be one of the main pathways through which microglia act to increase lamina I nociceptive neuron excitability to cause central sensitization. This mechanism has also been postulated to mediate the development of morphine hyperalgesia, as previously mentioned

(Ferrini et al., 2013). It is important to note, though, that this mechanism is sex specific (Sorge et al., 2015).

Other purinergic receptors have been implicated in the development of chronic pain. Genetic variability in P2X7 pore formation is a determinant of chronic pain susceptibility in both animal models and humans (Sorge et al., 2012). Proper pore formation of P2X7 receptors is thought to mediate the downstream effects that contribute to hypersensitivity, such as the release of IL-1b and ATP from microglia. Improper pore formation of the P2X7 channel can lead to a phenotype that is less susceptible to the development of chronic pain (Sorge et al., 2012). Inhibitors of P2X7 receptors have also been shown to reduce pain (Honore et al., 2006). Other studies examining purinergic receptors and pain have highlighted the involvement of the P2Y12 subtype of purinoreceptor in neuropathic pain (Tsuda and Inoue, 2016). For a complete review of purinergic signaling between neurons and microglia in chronic pain, see (Tsuda and Inoue, 2016).

The role of microglia in autoimmune disease associated pain has also been explored with several studies demonstrating that microglial activation contributes to neuropathic pain in EAE/MS (Khan, Gordon, Woodruff, & Smith, 2015; Olechowski et al., 2009; Zhu et al., 2013). Evidence suggests that a reduction in microglial activation is associated with a reduction in pain in EAE (Khan et al., 2015). The common pathways described above, such as the factalkine signalling pathway, have also been implicated in MS-related pain (Schmitz et al., 2013; Zhu et al., 2013). Microglial activation has also been implicated in pain associated with Guillain-Barré syndrome and rheumatoid arthritis (Luongo et al., 2008; Zhang et al., 2008; Christianson et al., 2010; Yang et al., 2014). It has also been found that pain in a mouse model of Guillain-Barré was associated with both the fractalkine pathway and an upregulation of P2X4 receptors, leading to the activation of spinal microglia (Luongo et al., 2008; Zhang et al., 2008). These studies demonstrate not only that microglial activation is important for the development of chronic pain in general, but that microglial activation is also associated with pain in autoimmune diseases.

The research outlined above demonstrates the connection among the activation of the immune system, chronic pain, and autoimmune diseases. Although a substantial amount of research in this area has been performed, there is still much to be learned to gain a better understanding of the underlying causes of chronic pain in autoimmune diseases. For example, the prevalence of autoimmune diseases is often higher among women than men, and this is

especially important given recent findings demonstrating significant sex differences in the mechanisms mediating chronic pain. (Sorge et al., 2015) found that different immune cells mediate pain hypersensitivity associated with peripheral nerve injury in male and female mice. These studies suggest that females rely on a T-cell mediated pathway, whereas males rely on a microglia mediated pathway. In addition, given the large variety of autoimmune disorders and their varying pathologies, it is unlikely that T-cell and microglial activation alone will account for chronic pain in these conditions. In line with this, new areas of research have been emerging. One such area, autoantibodies in pain and autoimmune disease, is discussed below.

New Insights into Autoimmune Disease-Related Chronic Pain: Autoantibodies

A consistently growing body of evidence implicates autoantibodies in pathological pain states. As mentioned, an autoantibody is an antibody produced by the immune system that is directed against an individual's own protein as opposed to an exogenous protein or pathogen (Mosby's Medical Dictionary, 2007). Studies have recently begun to show that autoantibodies not only contribute to disease pathology in autoimmune diseases but may also cause the associated pain states by acting against specific neuronal and glial proteins (Xiao et al., 1997; Schulte et al., 2006; Irani et al., 2010; Tamburin et al., 2014; McMahon et al., 2015). Here I will discuss how autoantibodies are produced and how they are thought to cause pathological pain. I will then discuss the role of autoantibodies in three different autoimmune diseases.

Autoantibody Production

Antibodies are normally produced by B cells in two different ways, randomly and in response to foreign proteins or substances in the body. Although autoantibodies can be produced randomly in the body, the immune system is designed to recognize and ignore the body's own proteins, cells, and tissues. However, occasionally these antigens are not recognized as "self," leading to the production of pathological autoantibodies. It is thought that the development of autoantibodies in autoimmune diseases may be linked to a loss of B-cell tolerance (Pillai et al., 2011; Giltiay et al., 2012).

There are normally two checkpoints during B-cell development to eliminate autoreactive B cells: a central checkpoint and a peripheral checkpoint (Meffre and Wardemann, 2008; Pillai et al., 2011; Giltiay et al., 2012). At the central checkpoint, possible autoreactive B

cells are eliminated through receptor editing mechanisms and deletion. Receptor editing involves rearranging the Œ and/or k light chain genes to generate new B cells that have a novel light chain that is no longer autoreactive (Pillai et al., 2011). B cells that pass the central checkpoint in development then enter the peripheral blood stream and red pulp of the spleen. At this point, B cells with autoreactivity are normally eliminated as they mature via various mechanisms such as anergy (a lack of immune responsiveness), clonal deletion (deactivation of B cells after expression of receptors for self-antigens), antigen specific inhibition by Tregs in a major histocompatibility complex (Samuels et al., 2005; Yurasov et al., 2005, 2006; Menard et al., 2011) class II- and CD40L-(Menard et al., 2011) dependent manner, clonal inhibition by the Siglec/SIaE pathway (prevents activation of B cells), or inhibition mediated by FcgRllb (Pillai et al., 2011). However, these checkpoints can be disrupted, causing the production of autoantibodies. Some weakly self-reactive B cells can fail to induce receptor editing, deletion, or anergy and therefore can mature and differentiate into follicular or marginal zone B cells, leading to autoimmunity (Pillai et al., 2011). Recent studies have indicated that different genetic polymorphisms associated with genes involved in regulating these checkpoints can be disrupted in autoimmune diseases (Arechiga et al., 2009; Menard et al., 2011; Giltiay et al., 2012; Rhee and Veillette, 2012). In addition, deficits in the central and peripheral checkpoints have been observed in patients with rheumatoid arthritis and systemic lupus erythematosus (Samuels et al., 2005; Yurasov et al., 2005, 2006; Menard et al., 2011). Overall, there are many mechanisms by which autoantibodies can develop and contribute to autoimmunity.

Role of Autoantibodies in Pathological Pain

One of the main ways in which autoantibodies are thought to contribute to pathological pain states is through alterations in voltage-gated ion channels and the consequent effects on the excitability of nociceptors. Autoantibodies to components of the voltage-gated potassium channel complex (VGKCC) have been implicated in autoimmune-associated pathological pain. VGKCCs are multimolecular membrane protein complexes in the nervous system that are critical in shaping action potentials in CNS neurons, controlling firing patterns, and modulating neurotransmitter release (Schulte et al., 2006). The VGKCCs Kv1.1 and Kv1.2 are located in the juxtaparanodal region of myelinated axons and are thought to suppress neuronal excitability. Therefore, interference with the localization and function of these

VGKCCs could lead to hyperexcitability (Schulte et al., 2006; McMahon et al., 2015). Autoantibodies against VGKCCs do not actually bind to the channel itself but rather to its associated proteins: leucine-rich glioma-inactivated protein I (LGI1), which is expressed in presynaptic terminals, or contactin-associated protein-like 2 (CASPR2), which is found in the juxtaparanodes (Irani et al., 2010). A study examining the effect of autoantibodies on these two proteins showed that CASPR2 was necessary for the localization of VGKCCs to the juxtaparanodes and suggested that binding of antibodies to CASPR2 could result in a downregulation of the CASPR2/Kv1.1/1.2 complexes on peripheral nerve axons, leading to neuropathic pain (Irani et al., 2010). The work examining the role of autoantibodies in VGKCCs has mostly been done in Morvan's syndrome, an autoimmune syndrome characterized by neuromyotonina, insomnia, and autonomic dysfunction. Morvan's syndrome is also commonly associated with pain. One study indicated that 50% of patients with Morvan's syndrome and VGKCC autoantibodies experienced pain, and in a small subgroup of these patients, pain was the only symptom (Klein et al., 2012). The patients all had idiopathic pain, thus this study demonstrated a potential biological basis for this pain (Klein et al., 2012). Furthermore, CASPR2 IgG was associated with pain in these patients, identifying it as a possible new therapeutic target (Klein et al., 2012). Although to date the role of VGKCCs has been explored mostly in Morvan's syndrome, this research has provided the field with promising new targets (e.g., CASPR2) to help manage autoimmune-associated chronic pain. Further research will have to be done to examine the role of ion channel autoantibodies in other pathological pain states.

Another way in which autoantibodies may mediate pathological pain is by causing structural damage to somatosensory neurons. The autoantibodies involved in the development of both Guillain-Barré syndrome and neuromyelitis optica (NMO) have been shown to cause structural damage (Xiao et al., 1997; Hinson et al., 2008). Autoantibodies in Guillain-Barré syndrome are specific for gangliosides in peripheral nerves and have been linked to the development of pain. One study showed that a monoclonal antibody treatment against the ganglioside GD2 for neuroblastomas caused pain and mechanical allodynia as a side effect (Xiao et al., 1997). This was thought to occur through alteration of the functional properties of small sensory fibers, causing increased ectopic activity and a reduction in mechanical thresholds (Xiao et al., 1997). Another study also measured death of cultured DRG neurons after incubation with autoantibody-positive serum from patients and showed that, after 2 days of incubation,

approximately 95% of the cultured neurons had died, especially large-diameter neurons (Ohsawa et al., 1993). The authors concluded that a loss of large diameter neurons in the DRG could contribute to sensory polyneuropathies in Guillain-Barré syndrome and therefore might contribute to the development of pain symptoms as well (Ohsawa et al., 1993). Additionally, axonal damage of unmyelinated and myelinated nerve fibers has been correlated with pain severity, and serum from autoantibody-positive patients with Guillain-Barré is cytotoxic to cultured sensory neurons (Ohsawa et al., 1993; Ruts et al., 2012). Overall, these studies provide a link between autoantibody-mediated damage and sensory dysfunction in Guillain-Barré syndrome.

The link between autoantibodies and pain in neuromyelitis optica (NMO) is similar to that seen in Guillain-Barré syndrome and involves structural damage to the axon. Approximately 75% of NMO cases are associated with autoantibodies against aquaporin 4 (AQP4), a water channel highly expressed on astrocytic foot processes (Lennon et al., 2005; McMahon et al., 2015). It is thought that binding of AQP4 autoantibodies leads to internalization of the channel, which can contribute to disease pathology (Hinson et al., 2008). In addition, serum from autoantibody-positive patients has been shown to trigger the production of chemokines, cytokines, and other stress factors, contributing to the generation of pathological pain (Howe et al., 2014). Autoantibodies against AQP4 may impair glutamate transport through a downregulation of glutamate transporters (Hinson et al., 2008). This disruption of glutamate homeostasis could potentially increase neuronal excitability by preventing the transport of glutamate needed to produce GABA in inhibitory interneurons, although more research is needed to support this theory (Hinson et al., 2008; McMahon et al., 2015). More work must be done to link AQP4 autoantibodies to pathological pain in NMO, but these studies give us insight into novel pain pathways and mechanisms.

Overall, the research regarding autoantibodies in Guillain-Barré syndrome and NMO demonstrates that autoantibodies may contribute to pathological pain by causing tissue damage in these diseases. The next few sections will examine new research looking at the role of autoantibodies in pathological pain associated with complex regional pain syndrome, Sjogren's syndrome, and rheumatoid arthritis because these three diseases exhibit unique ways in which autoantibodies may contribute to the development of pathological pain.

Autoantibodies in Complex Regional Pain Syndrome

Complex regional pain syndrome (CRPS) is an autoimmune disease that usually occurs after trauma to a limb and typically affects the injured limb (Tajerian and Clark, 2016). It is important to note that the extent of the injury does not affect the likelihood of developing CRPS and that immobilization (e.g., a cast) can also be a risk factor for its development (Veldman et al., 1993; Terkelsen et al., 2008). CRPS is painful, disabling, and often chronic in nature. Symptoms include sensory, motor, and autonomic nervous system dysfunction; cognitive deficits; changes in mood; anxiety; bone demineralization; changes in skin growth; and vascular dysfunction (Tajerian and Clark, 2016). CRPS can be divided into two different subtypes, CRPS type I and type II. Type I is CRPS without evidence of a major nerve lesion, whereas with type II there is evidence of a nerve lesion (Harden et al., 2007). Although there was previously some debate in the field on whether both subtypes experienced neuropathic pain—because type I, by definition, presents without nerve damage—new research indicates that both patient subtypes experience similar somatosensory disturbances. With pain profiles that are often reflective of neuropathic pain symptoms, such as the development of central sensitization (Gierthmühlen et al., 2014; Reimer et al., 2016). Other studies have also shown that, although CRPS type I patients often show some recovery within 6–13 months, some patients still experience chronic pain and disability (Bean et al., 2014).

CRPS was originally thought to be a response to sympathetic nervous system dysfunction and neurogenic inflammation. However, in recent years, evidence has come to light demonstrating that CRPS is most likely an autoimmune disease (Kohr et al., 2011; Goebel and Blaes, 2013; Dubuis et al., 2014). Some researchers have even proposed changing the name of a subgroup of patients with causative autoantibodies to "injury-triggered regional– restricted, autoantibody-mediated autoimmunity with a minimally destructive course" (IRAM;(Goebel and Blaes, 2013)). Previous work exploring CRPS has linked pain to spinal microgliosis and increased substance P, CGRP, and cytokine release (for a review of these mechanisms see (Tajerian and Clark, 2016)). Work over the past decade, however, has shown that CPRS has autoimmune components, the most prominent being autoantibodies that bind to and activate M2-muscarinic (M2) and β2-adrenergic receptors (β2-AR; (Kohr et al., 2011; Goebel and Blaes, 2013; Dubuis et al., 2014; Goebel, 2016)). For example, inhibitory M2 receptors have been shown to be upregulated in the DRG after nerve injury, which may represent an endogenous

analgesic mechanism (Hayashida et al., 2006). It is also thought that if the receptor expression is changed locally by autoantibody activation it may contribute to the restriction of CRPS to one limb, especially insofar as increases in M2 expression after injury have been found only on the ipsilateral side (Hayashida et al., 2006; Kohr et al., 2011). M2 receptors are known to regulate nerve excitability and neuropeptide release in inflammatory conditions, which may contribute to pain, but more research is needed to elucidate these mechanisms fully (Kohr et al., 2011). β2-ARs can be found on immune cells and can be activated by catecholamines, leading to the production and release of inflammatory cytokines by inhibiting the release of IL-12 and stimulation of IL-10, an anti-inflammatory cytokine (Maestroni, 2006). How activation of these receptors, which seem to be involved mostly in endogenous analgesic mechanisms, contribute to disease pathology and pathological pain is still not fully understood. However, one study has shown that genetic variation in the β2-AR is linked to chronic pain, and, more importantly, it has also been shown that serum IgG from CRPS patients can cause pain when passively transferred to an animal model (Hocking et al., 2010; Tékus et al., 2014). Finally, depletion of B cells in a mouse model of CRPS has been shown to reduce nociceptive symptoms, again suggesting a role for autoantibodies in CRPS that is not yet understood (Li et al., 2014). Although the role of autoantibodies remains to be fully elucidated, these results provide insight into additional pathways by which autoantibodies may contribute to pathological pain, the activation of G-coupled protein receptors.

Autoantibodies in Sjogren's Syndrome

Sjogren's syndrome is a systemic autoimmune disorder that targets exocrine gland epithelia, especially cells of the lachrymal and salivary glands (Vitali and Del Papa, 2015). Sjogren's syndrome is characterized by the loss of glandular function causing dry eyes and mouth but often has secondary clinical manifestations that can affect the joints, lungs, kidneys, small vessels, and central and peripheral nervous systems, ultimately leading to pain and fatigue (Chai et al., 2005; Moreira et al., 2015; Vitali and Del Papa, 2015). Autoantibodies against Ro60 and Ro52, also known as anti-SSA, and anti-SSB, have been identified in patients with Sjogren's syndrome (Ching et al., 2011). The SSA antigen is made of both Ro52, a member of the tripartite motif family of proteins, and Ro60, a protein that binds small cytoplasmic RNAs (Ching et al., 2011). The SSB antigen is made of a single 47-kDa protein known as La (Ching et al., 2011).

Although it is known that these antibodies play a role in disease development, they do not appear to be linked to Sjogren's syndrome associated pain. In fact, two studies examining the connection between autoantibodies in SS and pain have shown that it is seronegative patients who report the most pain (Segal et al., 2013; ter Borg and Kelder, 2014). This is interesting given the studies described above in which the presence of autoantibodies is often associated with pain. However, the development of pain in Sjogren's syndrome is still poorly understood, and work exploring new autoantibodies involved in Sjogren's syndrome is emerging (Baer et al., 2015). For example, one study indicated that an antibody to nuclear autoantigen of 14 kDa (NA14) is involved in Sjogren's syndrome and that some patients who were anti-ND14-positive were also negative for anti-SSA/SSB (Nozawa et al., 2009). This suggests that there are additional autoantibody targets to be explored to better understand the development of pathological pain in Sjogren's syndrome. On the other hand, it is possible that in those seropositive patients the antibodies also activate some sort of endogenous analgesic mechanism that is protective against pathological pain. In either case, more research is needed to fully elucidate pain pathways in Sjogren's syndrome.

Autoantibodies in Rheumatoid Arthritis

Rheumatoid arthritis is a systemic chronic inflammatory autoimmune disease with extensive synovitis, erosion of the articular cartilage, and marginal bone and joint destruction (Aletaha et al., 2010; Conigliaro et al., 2016). Multiple autoantibodies have been linked to the development of rheumatoid arthritis and there seems to be a correlation between antigen level and disease progression/severity (for a complete review see (Conigliaro et al., 2016). Several studies have begun to link autoantibodies involved in rheumatoid arthritis to pain. Bone destruction caused by the binding of autoantibodies to osteoclasts leads to the subsequent release of CXCL1/IL-8, disease progression, and possibly pain (Seeling et al., 2013; Krishnamurthy et al., 2016). A recent study has expanded on this work, demonstrating that administration of either human or murine anti-citrullinated protein antibody (ACPA), an autoantibody implicated in rheumatoid arthritis, to mice caused nociceptive behavior for up to 28 days post-injection (Wigerblad et al., 2016). This pain was found to develop in the absence of inflammation, suggesting that the pain was entirely antigen induced (Wigerblad et al., 2016). It is thought that this increase in nociceptive behavior was due to the activation of osteoclasts and the release of the nociceptive

chemokine CXCL1/IL-8, which can activate sensory neurons (Wigerblad et al., 2016). In addition, this pain could be reversed with the IL-8 receptor antagonist reparixin (Wigerblad et al., 2016). This study provides new and exciting evidence that autoantibodies may directly trigger pathological pain in rheumatoid arthritis. However, it is important to note that this is not likely the only cause of pain in rheumatoid arthritis because patients may have ACPA autoantibodies for up to a decade before clinical symptoms appear. Furthermore, disease modifying drugs that help alleviate pain do not necessarily reduce levels of ACPA (Sokolove and Pisetsky, 2016). However, a better understanding of how ACPA and the immune system interact to alter immune modulating factors such as IL-8 could lead to the development of better pain-targeting therapeutics in rheumatoid arthritis.

Overall, a substantial body of evidence links the immune system, autoimmune disease, and development of pathological pain. Not only do "classical" immune components such as T-cells and microglial activation apply to autoimmune disease-related pain, but new research implicates autoantibodies as a possible mechanism mediating pain associated with these disorders. A better understanding of how autoantibodies are involved in the pathological pain states associated with autoimmune disorders could lead to promising new therapeutic targets. In fact, using IgG for the treatment of chronic pain has already shown promise in several autoimmune diseases, such as CRPS and Sjogren's syndrome (Tamburin et al., 2014). In addition, as previously mentioned, downstream targets of autoantibody actions, such as IL-8 in rheumatoid arthritis, may also prove to be useful in alleviating pain. Overall, the links between autoimmunity and pain are proving to be an interesting and fruitful area of research.

One area of particular interest to this thesis, is the potential role of autoantibodies in the development of chronic pain in MS. Unfortunately, there has been little research in this area as there is still no agreement on the exact autoantibodies that are involved in the pathology of MS. Furthermore, the exploration of mechanisms unique to chronic pain in the context of MS is still relatively new compared to chronic pain in general. Therefore, the chapters in the current thesis were designed to try and understand common mechanisms between the development of MS and chronic pain in MS. The section below outlines our current knowledge on the mechanisms behind MS progression and the development of pain in the disease.

Multiple Sclerosis and its Associated Chronic Pain

Multiple sclerosis is classically described as a chronic neurodegenerative demyelinating autoimmune disease of the central nervous system. Approximately 2-3 million people are thought to live with MS globally, making this disease a world-wide health issue (Thompson et al., 2018). Prevalence rates in Canada are extremely high compared to other countries, and rates have been increasing over time in several provinces in Canada (Alonso and Hernán, 2008; Marrie et al., 2010, 2013; Kingwell et al., 2015). Thus, not only is MS a world-wide health issue, but also one that is close to home. There are three types of MS: relapsing remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS). RRMS is defined by relapses separated by disease free intervals without progression and accounts for about 90% of patients (Makris et al., 2014). PPMS is defined by a lack of disease free intervals and continuous progression (Makris et al., 2014) . This is the most disabling type of MS. Finally SPMS begins after RRMS disease when a progressive pattern of disability develops (Makris et al., 2014).

The primary symptom of MS is increasing motor dysfunction over time, yet many patients experience a host of secondary symptoms such as cognitive dysfunction, fatigue, bladder dysfunction, and chronic pain (Crabtree-Hartman, 2018). Chronic pain as a common secondary symptom of MS is of particular interest given that approximately 50% of patients develop pain during their disease course (Kalia and OConnor, 2005; O'Connor et al., 2008; Solaro and Messmer Uccelli, 2011; Murphy et al., 2017). While pain has been reported in the clinical population for some time, it was not until the past decade that chronic pain was systematically examined in the most common animal model of MS: experimental autoimmune encephalomyelitis (EAE). The EAE model is induced by injecting either rats or mice with myelin antigen in combination with pertussis toxin and Complete Freud's Antigen (CFA) to cause a heightened immune response. This combination of injected antigens causes the production of myelin antibodies through a T-cell mediated pathway leading to demyelination and inflammation in the CNS. In addition, rodents demonstrate reliable ascending paralysis starting at the tail. Furthermore, the myelin antigen and adjuvant doses can be titrated to alter the severity of the model or to create relapsing remitting vs chronic models (Berard et al., 2010). Impaired nociception was first seen in a 1986 study where it was found that there was sensory loss in the tail with ascending paralysis (Pender, 1986). However, given that nociceptive behavior was measured at the time of paralysis, it is difficult to say whether or not the paralysis masked any

potential nociceptive processing. Thus, more recent studies have explored different time points and found that chronic pain associated with MS can be modeled in EAE (Aicher, Silverman, Winkler, & Bebo, 2004a; Olechowski et al., 2009; Rahn, Iannitti, Donahue, & Taylor, 2014). While chronic pain in MS can be modeled, the mechanisms of how it develops are still not fully understood. To date studies have shown that several mechanisms appear to be involved in the development of chronic pain in MS, such as increased inflammation and reactive gliosis in the CNS, increases in the levels of inflammatory cytokines, changes in neurotransmitter levels, and alterations in transporter proteins critical for the maintenance of synaptic homeostasis (Begum, Zhu, Cortes, MacNeil, & Namaka, 2013; Lisi et al., 2012; Melanson et al., 2009; Olechowski, Tenorio, Sauve, & Kerr, 2013; Camille J Olechowski et al., 2009; Olechowski et al., 2010; Potter et al., 2018; Zhu et al., 2013). While these studies provide insights into the mechanisms underlying chronic pain in MS, more work needs to be done to fully understand these pathways. The sections below outline how new research in the areas of oxidative stress, cytokine changes, and neuroactive steroids not only contribute to MS pathology, but also MS related pain.

Studies to date have also failed to account for variables that may influence MS related pain and MS pathology more generally. For instance, sex, as in chronic pain, is also an important variable to consider in MS research. It is well established that there is a higher prevalence of MS in the females and that this number is increasing compared to males (Orton et al., 2006; Thompson et al., 2018). Furthermore, it is known that while female MS patients are more common, the risk of long-term disability is higher in male patients (Shirani et al., 2012; Voskuhl et al., 2018). In addition, several studies have explored the role of sex differences in the immune response to EAE induction (Dunn et al., 2010, 2015, Zhang et al., 2012a, 2015). These studies demonstrated that different T-cell profiles can differentially contribute to disease severity/susceptibility in MS (Dunn et al., 2007; Zhang et al., 2012a). Androgen levels in male mice cause an increase in the expression of the nuclear factor receptor, peroxizome proliferator-activated receptor alpha (PPARα) and decrease PPARγ expression, leading to a reduction in the production of the cytokines TNFα and INFγ, but increasing the levels of IL-17. This increase in PPARα has been linked to a protective effect for males with regard to autoimmune diseases, while the lack of androgen activated PPARα in females has been associated with an increase in autoimmune diseases such as MS (Dunn et al., 2007; Zhang et al., 2012a). Interestingly, these differences in PPAR activation have also been associated with sex differences in immune

mediated nociceptive pathways. Nociceptive hypersensitivity in male mice is thought to be mediated through microglia activation where as in females it is through T-cell activation (Sorge et al., 2015). These studies again highlight the importance of considering sex as a variable.

Major Pathways Contributing to Damage in MS & MS Related Chronic Pain

There are multiple pathways that contribute both to disease pathology and chronic pain in MS. The sections below outline some of the new areas of research in these topics, and outline how chronic pain in MS may be unique compared to non-disease related chronic pain conditions.

Oxidative Stress in MS & MS Related Pain

Oxidative stress can be defined as an imbalance between the production of free radicals and the ability of the body to detoxify their harmful effects via neutralizing antioxidants. A reduction in oxidative stress in many diseases is important as free radicals contribute to damage that leads to disease progression. Protection from free radicals comes from the antioxidant defense system which is mainly comprised of free radical scavengers and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and glutathione (GSH). Reactive oxygen species, on the other hand, are produced by the cytosol, mitochondria, lysosomes, peroxisomes, and the plasma membrane under both normal and pathological conditions.

The CNS is thought to be particularly susceptible to oxidative stress due to its high rate of oxygen consumption. Oxidative stress in MS is thought to contribute to the progression of the disease over time by promoting neurodegeneration. MS patients have been shown to have high levels of lipid peroxidation, a measure of oxidative stress, in the CSF when compared to healthy controls (Calabrese et al., 1998). An additional study of RRMS patients found that traditional molecules involved in regulating the redox balance were disrupted as seen again by increases in lipid peroxidation and protein carbonylation (Polachini et al., 2016). Furthermore, the study went on to show that there was a decrease in the SOD enzyme and increased DNA damage, all classic hallmarks of oxidative stress (Polachini et al., 2016). Another study demonstrated that lipid peroxidation precedes the inflammatory response in RRMS, suggesting that oxidative stress may be an early event in MS in addition to being directly linked with inflammation and neurodegeneration (Wang et al., 2014).

Research has also found that oxidative stress can contribute to the neuroinflammatory response by mediating T-cell changes in neurodegenerative disease (Solleiro-Villavicencio and Rivas-Arancibia, 2018). It has been proposed that oxidative stress can inhibit the responses of anti-inflammatory Th2 T-cells (Solleiro-Villavicencio and Rivas-Arancibia, 2018). For example, a study has observed that mitochondrial inhibitors of reactive oxygen species (ROS) lowered differentiation of T-cells to the Th17 phenotype in a mouse model of arthritis, suggesting that different levels of ROS can modulate T-cell phenotype (Zhi et al., 2012). Given that EAE is a predominately T-cell mediated disease, it is possible that similar changes are occurring in MS, though more research is needed to better understand the interaction between T-cells and oxidative stress in MS (Solleiro-Villavicencio and Rivas-Arancibia, 2018).

Increased oxidative stress can also lead to mitochondrial dysfunction, paradoxically triggering the production of more ROS and subsequent oxidative stress in a vicious cycle. This process is thought to occur when ROS and reactive nitrogen species (RNS) diffuse across the mitochondrial membrane and compete with oxygen molecules for binding sites on components of the electron transport chain, such as the cytochrome c oxidase enzyme (Haider, 2015). This ultimately decreases respiratory chain function and can lead to the development of "respiratory deficient" neurons, which are often seen in the lesions of patients (Mahad et al., 2008; Haider, 2015). Both ROS and RNS can also cause covalent modification and mutations in mitochondrial DNA that also inhibit the efficiency of oxidative phosphorylation and increase production of ROS, completing the vicious circle (Yakes and Van Houten, 1997; DiMauro et al., 2013). Evidence of mitochondrial DNA deficits have also been seen in tissue from MS patients and are proposed to contribute to neurodegeneration (Dutta et al., 2006; Campbell et al., 2011) . Further evidence of increased oxidative stress and subsequent mitochondrial dysfunction comes from studies where detoxification of ROS reverses both markers of mitochondrial and axonal injury in EAE (Nikić et al., 2011). Taken together these studies demonstrate that oxidative stress is a hallmark feature of the pathophysiology of MS.

Interestingly, oxidative stress has also been implicated in the development of chronic pain. Studies in patients with fibromyalgia found increased ROS, impaired mitochondrial function and reduced ATP in the muscle and neural cells of patients (Meeus et al., 2013). A meta-analysis of the antioxidant therapy for chronic pancreatitis also found that this therapy reduced pain in this disease, demonstrating a role for oxidative stress in pancreatitis pain

(Rustagi and Njei, 2015; Talukdar et al., 2015). Oxidative stress is also thought to play a major role in the development of diabetic and chemotherapy-induced neuropathies (Bordet and Pruss, 2009; Araki and Nishikawa, 2010; Reichling and Levine, 2011). It has also been shown that antioxidant administration can reduce hyperalgesia in these conditions (Joseph et al., 2008). While these studies demonstrate that oxidative stress is likely involved in the development of chronic pain, there has been little research to date looking at oxidative stress in MS related pain. More research is therefore needed to determine if oxidative stress plays a role in the development of chronic pain in MS.

Cytokines in MS & MS Related Pain

As mentioned above, the development of MS and other autoimmune diseases is thought to be due in part to an imbalance in T-cell Th1/Th2 immunity (see above for details). This is also seen in studies showing the progression and initial disease stages of MS are linked to a Th1/Th2 imbalance (Begolka et al., 1998; Sospedra and Martin, 2005). The Th17 cell population and its related cytokines (IL-17, IL-17F, and IL-22) are also involved in the development of MS through their ability to promote inflammation and autoimmunity (Steinman, 2010; Moynes et al., 2014). Cytokines released from T-cells can activate microglia and also directly act on neurons, promoting increased immune cell infiltration (for a complete review see Moynes et al., 2014). In addition, infiltrating macrophages and activated microglia also produce cytokines. Reactive macrophages/microglia can also be roughly categorized into two different phenotypes: M1 and M2 phenotypes. Similar to the Th1/Th2 phenotypes, M1 macrophages are thought to be pro-inflammatory and M2 to be anti-inflammatory due to their release of the anti-inflammatory cytokines Il-4, IL-10, IL-13, IL-33 and TGF-β. It is also thought that M1 macrophages/microglia promote tissue damage, demyelination, and neuronal death in the CNS, while M2 macrophages/microglia help promote tissue repair and the resolution of inflammation (Chu et al., 2018). Changes in serum levels of patient cytokines have also been observed, with one study reporting increased levels of Th17-related cytokines in MS (Li et al., 2017). Overall, it is thought that the increase in cytokines in MS/EAE lead to an increase in immune cell infiltration, a breakdown of the blood-brain barrier (BBB) integrity, inflammation and tissue damage (Palle et al., 2017).

It is important to note however, that cytokines are not always categorized neatly into pro- or anti-inflammatory profiles in EAE. An example of this is the cytokine TNFα in the pathogenesis of EAE and MS. Pre-clinical studies of the role of TNFα in the development of MS found that patients had elevated CSF levels of this cytokine and that it was correlated with disease severity and progression (Maimone et al., 1991; Sharief and Hentges, 1991). Additionally, it was found that TNFα neutralizing antibodies prevent development of symptoms in the adoptive transfer model of EAE and that treatment of mice with TNFα antibodies at disease onset suppressed the development of disease (Ruddle et al., 1990; Inoue et al., 1996). Overall the pre-clinical evidence indicated that a reduction of TNFα levels in patients should be beneficial. However, when a clinical trial with a recombinant TNF receptor, lenercept, was tested, it was found to exacerbate disease pathology in patients (Anon, 1999; Palle et al., 2017). Interestingly, a similar effect was found in exploring the role of the interferon (INF) family of cytokines. Previous studies had shown that increasing levels of INFβ was beneficial, but a study of RRMS patients where increasing INFγ levels was detrimental (Panitch et al., 1987). This demonstrates that cytokines in MS are likely more complex than originally thought and that they have many different roles in neuroinflammation.

Several cytokines, including TNFα, have been implicated in the development of MS related pain. Levels of TNFα are increased in the DRG and spinal cord of EAE mice who demonstrate enhanced nociceptive behaviours (Melanson et al., 2009; Begum et al., 2013; Rodrigues et al., 2016). Additional studies have shown that increased levels of IL-17 and INFγ are also associated with the development of nociceptive hypersensitivity in EAE (Lisi et al., 2012; Schmitz et al., 2013). Increased levels of IL-1β are also found to be associated with the development of pain in EAE (Olechowski et al., 2013; Rodrigues et al., 2016). Therapies designed to target cytokines associated with development of chronic pain have also been shown to be effective. One study used a cannabinoid receptor (CB2) agonist to reduce cytokine levels and saw analgesic effects of the agonist (Alberti et al., 2017). Another study used gene therapy to increase the levels of the anti-inflammatory cytokine IL-10 and also found improvements in nociception in the EAE model (Sloane et al., 2009). While these studies demonstrate that cytokines are involved in the development of chronic pain in MS, more work is needed to clarify the exact mechanisms that drive this nociceptive behaviour. Furthermore, more work is needed

to translate this pre-clinical research into clinical trials to better manage chronic pain in MS patients.

Neuroactive Steroids in MS & MS Related Pain

Neuroactive Steroids (NASs) are rapidly acting steroids that produce nongenomic effects by acting as allosteric modulators at neurotransmitter receptors. NASs typically act at the NMDA subtype of glutamate receptors and/or gamma-aminobutyric acid-A (GABA-A) receptors (Tuem and Atey, 2017). NASs may be synthesized from cholesterol in the brain or they can come from the periphery. Some NASs have also been reported to have anti-inflammatory actions and have been proposed to play a role in a number of neurologic, psychiatric and gastrointestinal disorders (Giatti et al., 2012; Tuem and Atey, 2017). In addition to studies reporting changes in levels of NASs in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, changes in MS have also been reported (Giatti et al., 2012). A clinical study examining CSF levels of NAS in patients found increased levels of pregnenolone (PREG) and dehydroepiandrosterone (DHEA) (Orefice et al., 2016). Interestingly, these increases were associated with active disease state in females and the presence of gadolinium enhancing lesions in male patients (Orefice et al., 2016). This suggests that increases in PREG may be promoting disease progression and/or severity. Furthermore, these changes were regulated by disease state in the patients studied (Orefice et al., 2016). Similar disease state dependent changes in NAS levels can also been seen in the EAE model. For example, in the acute stage of the EAE, there are decreased spinal cord levels of progesterone (PROG) and isopregnanolone (ISO) (Caruso et al., 2010; Giatti et al., 2010). However, NAS levels in the spinal cord do not appear to change at the chronic stage of EAE (Caruso et al., 2010; Giatti et al., 2010). Another study exploring NAS levels found that males showed decreased testosterone in progressive MS compared to RRMS suggesting that different NASs might have different roles in disease progression (Savettieri et al., 2004; Caruso et al., 2010; Giatti et al., 2010; Orefice et al., 2016).

A major NAS whose levels change in MS is allopregnanolone (ALLO). ALLO is generally thought to be neuroprotective, especially in MS (Noorbakhsh et al., 2014). For example, a study examining the levels of ALLO in the white matter of brains of patients with MS found decreased ALLO levels (Noorbakhsh et al., 2011). Interestingly sex differences in ALLO levels have also been reported. Female MS patients have reduced levels of ALLO in the CSF,

while levels of ALLO in males with MS is stable (Orefice et al., 2016). Additionally, administration of ALLO/ALLO ligands to mice with EAE reduced neuroinflammation and disease severity (Wang et al., 2002; Daugherty et al., 2013). Interestingly, changes in ALLO have been linked to chronic pain. However, this is a new area of research, so this study was not done in MS patients, but rather in patients with post-traumatic stress disorder. This study found that changes in ALLO levels are associated with post-exercise pain tolerance in patients with post-traumatic stress disorder who also have chronic pain (Scioli-Salter et al., 2016). This study indicates that in addition to their involvement in MS pathophysiology, NASs may regulate pain processing.

Summary & Purpose

Chronic pain is a complex disease associated with numerous pathophysiological changes, such as altered neurotransmission, oxidative stress, inflammation, and neuronal damage. Despite decades of research into the mechanisms of this condition, there are still no effective treatments for many types of chronic pain. One of the reasons for this could be lack of research into variables such as sex that greatly influence pain processing. As seen above, sex not only has a role in the development of chronic pain in general, but also in the development of chronic pain in specific chronic disease states such as MS. This is particularly seen in how sex influences the role of the immune system in the development of chronic pain and the role of sex in autoimmune pain. The involvement of different biological mechanisms in the development of chronic pain between the sexes can also directly impact therapeutic benefit of different treatments for pain. Therefore, the purpose of this thesis was to examine the role of sex in pain behaviors and treatments in different models of chronic pain.

In Chapter 1, I examined the role of sex and neurotransmitter levels in the formalin model. To do this, levels of 5-HT, NA, and GABA were pharmacologically manipulated in male and female mice who received formalin injections and nociceptive behaviors were observed.
Hypothesis 1: Formalin Pain is regulated by different neurotransmitter systems in male and female mice.

In Chapter 2, I began to explore the effect of sex on the benefits of voluntary wheel running in EAE. Differences in disease scores and disease markers (axonal damage, demyelination, immune cell infiltration, oxidative stress) were examined between male and female mice who had access to a running wheel.
Hypothesis 2: Voluntary wheel running will have beneficial effects in male and female mice with EAE.

In Chapter 3, I continued the study of voluntary wheel in male and female mice with EAE. Changes in the brain associated with voluntary wheel running were explored by assessing NAS levels in male and female mice.
Hypothesis 3: Neuroactive steroids in the cerebrum of mice with EAE will be modulated by voluntary wheel running.

In Chapter 4, I explored the effect of voluntary wheel running on nociceptive behavior in male and female mice with EAE. Changes in levels of peripheral cytokines levels and DRG excitability were also explored to examine effects of running and sex on nociceptive behavior associated with EAE.
Hypothesis 4: Voluntary wheel running will differently affect pain behavior in male and female mice with EAE.

Table 1. Autoimmune Diseases Associated with Pain

Autoimmune Disease	Disease Description	Associated with Pain?	Reference
Alopecia Areata	Hair loss in round patches	No	(Spano and Donovan, 2015)
Autoimmune Hemolytic Anemia	Characterized by shortened red blood cell survival and presence of antibodies against red blood cells, mediated by temperature	Yes (various)	(Packman, 2015)
Autoimmune Hepatitis	Progressive inflammatory liver disease of unknown etiology	Yes (abdominal)	(Jiménez-Rivera et al., 2015)
Dermatomyositis	Systemic rheumatic disease characterized by a skin rash and chronic muscle inflammation	Yes (muscle)	(Kishi et al., 2013)
Diabetes (Type 1)	Condition in which the pancreas ceases to produce insulin	Yes	(Downs and Faulkner, 2015)
Juvenile Idiopathic Arthritis	Most common form of joint swelling, heat and pain in children	Yes (joint)	(Schanberg et al., 2003)
Glomerulonephritis	Acute inflammation of the kidney	No	(Murtas and Ghiggeri, 2016)
Graves' Disease	Swelling of neck and protrusion of eyes due to hyperthyroidism	Yes (eye)	(Cardoso et al., 2012)
Guillain-Barré Syndrome	Autoimmune polyneuropathy disease attacking the peripheral nervous system	Yes (various)	(Yuki and Hartung, 2012)
Idiopathic Thrombocytopenic Purpura	Easy or excessive bruising or bleeding, possibly due to low platelet count	No	(Cines et al., 2009)
Myasthenia gravis	Autoimmune disease cause by complement-fixing antibodies against AChR characterized by severe muscle weakness	No	(Dalakas, 2015)

Autoimmune Disease	Disease Description	Associated with Pain?	Reference
Multiple Sclerosis	a demyelinating progressive inflammatory autoimmune disease	Yes(neuropathic/mixed)	(Pöllmann and Feneberg, 2008)
Pemphigus/ Pemphigoid	Autoimmune blistering disease of the skin	Yes (lesions)	(Rajan et al., 2014)
Pernicious Anemia	Vitamin B12 deficiency anesmia	Yes (various)	(Osborne and Sobczyńska-Malefora, 2015)
Polyarteritis Nodosa	Autoimmune disease causing spontaneous inflammation of the arteries	Yes	(De Virgilio et al., 2016)
Polymyositis	Autoimmune disease causing muscle weakness	Yes (chest)	(Danieli et al., 2016)
Primary Biliary Cirrhosis	Disease with inflammation and progressive destruction of small intrahepatic bile ducts	Yes (abdominal)	(Cheung et al., 2016)
Psoriasis	Chronic immune mediated skin disease with erythematous plaques and silvery scales	Yes (plaque)	(Lebwohl et al., 2014)
Rheumatoid Arthritis	Systemic inflammatory disease causing pain and joint destruction	Yes (joint)	(Suurmeijer et al., 2001)
Scleroderma/systemic sclerosis	Chronic autoimmune multisystem connective tissue disease with thickening and fibrosis of skin and internal organs	Yes (various)	(Racine et al., 2016)
Sjögren's Syndrome	Systemic autoimmune disease targeting exocrine gland epitheslia, especially the lachrymal and salivary glands	Yes (joint/muscle)	(Vitali and Del Papa, 2015)
Systemic Lupus Erythematosus	Chronic unpredictable autoimmune disease that can affect any healthy tissue	Yes (various)	(Greco et al., 2003)

Autoimmune Disease	Disease Description	Associated with Pain?	Reference
Vitiligo	Disease of pigment-producing cells that result in varying patterns and degrees of skin depigmentation	No	(Bretterklieber et al., 2014)
Granulomatosis with Polyangiitis (Wegener's disease)	Autoimmune disorder causing inflammation of blood vessels in nose, sinuses, throat, lung and kidneys	Yes (various)	(Tomasson et al., 2012)

Chapter 1: Manipulation of Neurotransmitter Levels has Differential Effects on Formalin-Evoked Nociceptive Behaviour in Male and Female Mice

1.0 Introduction

It is well established in clinical populations that differences exist in the prevalence rates of persistent pain between the sexes. Females are reported to have higher prevalence rates for most persistent pain conditions (Berkley, 1997; Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; Greenspan et al., 2007; Vincler, Maixner, Vierck, & Light, 2001)]. Differences in acute pain sensitivity are also seen, with women reporting greater sensitivity to different noxious stimuli (Rocha-González et al., 2005; Gazerani et al., 2006; Greenspan et al., 2007; Fillingim et al., 2009). Sex differences are also found in animal models of pain and nociception (Craft, n.d.; Mogil et al., 2003; Berkley et al., 2006; Fillingim et al., 2009; Muñoz-Islas et al., 2014). Studies examining the underlying mechanisms of pain have found that males and females do not always respond in the same manner to many novel or classically prescribed analgesics (Craft, 2003; Mogil et al., 2003; Berkley et al., 2006; Fillingim et al., 2009; Mogil, 2012). Results from these studies suggest that different underlying mechanisms may contribute to male and female nociception.

Despite these findings, little research has been done to examine potential sex differences in the role of neurotransmitter systems in regulating pain and nociception. Neurotransmitters such as serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline (NA) have been implicated in pain modulation and many antidepressants that elevate the synaptic cleft levels of these neurotransmitters are considered first line treatment for certain types of chronic pain (Dworkin et al., 2007; Attal et al., 2010; Bohren et al., 2013; Sagheddu et al., 2015). It is well established that increases in the levels of 5-HT and NA in the spinal cord can potentiate anti-nociception (Yang et al., 2005; Roh et al., 2006; Greenspan et al., 2007; Hughes et al., 2013b; Rahn et al., 2014). It is also well established that γ-aminobutyric acid (GABA) is critical for regulating pain processing. Several studies have shown that reduced levels of GABA in the spinal cord are linked to the development of chronic pain (Moore et al., 2002; Zeilhofer et al., 2009).

However, little research has been done to examine how these three different neurotransmitters interact to modulate nociceptive responses. Studies of the relative effectiveness

of tricyclic antidepressants (TCAs) versus selective serotonin inhibitors (SSRIs) suggest that the TCAs are more effective at alleviating pain than the more specific SSRIs (Finnerup et al., 2010; Sagheddu et al., 2015). This indicates that there may be a significant benefit to altering multiple neurotransmitters systems at once. Also, given the well-described sex differences in several underlying mechanisms of pain processing, sex may also have a role in how the nervous system responds to changes in neurotransmitter levels with treatment.

In the current set of experiments, we used the formalin model to study how manipulating neurotransmitter systems affects nociception in male and female mice. Phenelzine (PLZ), a monoamine oxidase and GABA-transaminase inhibitor, and two analogues of PLZ were used to examine the effects of altering central nervous system levels of 5-HT, NA, and GABA together or separately on formalin evoked nociceptive behaviours. Our results describe the relative influence of each neurotransmitter on nociception and also characterize how male and female mice respond differently to these manipulations.

1.1 Materials & Methods

1.1.1Animal Care

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. Male and female 6-8 week old C57BL/6 mice (http://www.criver.com/products-services/basic-research/find-a-model/c57bl-6n-mouse) were used for all experiments. All animals were group-housed (5 mice per cage) in a controlled room with a 12-hour light/dark cycle and were given free access to standard food pellets and water. All animals underwent at least a week of handling prior to beginning experimental procedures to ensure that they were familiar with the researchers doing the testing. This handling period also ensured that mice had ample time to reach sexual maturity. Thus, age was not a factor in potential hormone differences, and sexual maturity in females was further confirmed by estrus cycle monitoring. Most of the behavioral testing was done by a female researcher (K.M.), and for testing done by a male researcher (K.T.), that male researcher was in the room with the mice for a 30 minute period prior to formalin testing to prevent any inhibition of nociceptive responses that have been observed in male mice after exposure to volatile androgens produced by male researcher (Sorge et al., 2014). This waiting period ensure that the mice acclimatize to the

presence of the male researcher. Gonadectomized mice and sham surgery mice were ordered from Charles River Canada and shipped after surgical clips had been removed. Animals were allowed two weeks to further recover and adjust to new housing facilities prior to testing.

1.1.2 Formalin Induction

The formalin test was used to examine nociceptive behaviour in both male and female mice (Dubuisson and Dennis, 1977). Formalin testing occurred 3 hours after mice had been pre-treated with either an active drug or vehicle solution (see below). Mice were tested during the light phase and testing was balanced to ensure that an equal number of mice were tested in the morning and afternoon. Each animal was allowed to habituate to the testing apparatus (a 25cm x 23cm x 15cm clear plexi-glass observation box with a raised platform) for 30 minutes prior to testing. Mice were then gently restrained and given a 30µl subcutaneous injection of 1% formalin (37% formaldehyde solution, Sigma, in 0.9% saline) in the plantar surface of one hind paw. After the injection, mice were placed back into the apparatus and observed for 30 minutes. During this period, the time spent engaging in nociceptive behaviours (licking and/or lifting/flinching of the injected paw) was monitored and timed (in seconds) in 5-minute interval blocks. The total time (in seconds) spent displaying nociceptive behaviours was then determined for the first phase (0-10 minutes) and second phase (10-30 minutes) of the assay. The investigator was able to assess both licking and lifting behaviours as scoring was done live with the investigator in the room. The investigator was also blinded to treatment group, as injections were done by another experimenter. Mice were randomly assigned to treatment groups in all experiments to ensure one cage did not receive all the same drug treatment.

1.1.3 Experiment One: An Examination of the Analgesic Properties of PLZ and PEH

Levels of GABA, 5HT, and NA were altered using PLZ and its active metabolite phenylethylidenehydrazine (PEH). Mice were pre-treated with either PLZ or PEH via intraperitoneal (i.p.) injection 3 hours prior to formalin testing. This time interval allows for the maximum increase in CNS levels of 5-HT, NA and/or GABA to occur (McManus et al., 1992; Musgrave et al., 2011a; McCarson and Enna, 2014). Male and female mice were divided into the following groups:

Naïve (n=4M, 5F): Naïve mice did not receive any pretreatment or formalin testing. Animals were euthanized and tissue removed without any experimental procedures to determine baseline levels of the neurotransmitters examined.

Environmental Control (n=5M, 6F): Animals underwent the same experimental protocol as vehicle treated animals (see below), but received a 30µl injection of 0.9% saline instead of formalin into one hind paw and no drug pre-treatment. This group was used to determine the time spent engaging in nociceptive behaviours that are associated with the hind paw injection procedure alone.

Vehicle (n= 5M, 5F): Animals received a single i.p. injection of bacteriostatic water prior to formalin testing.

PLZ (n= 12M, 12F): Animals received a single i.p. injection of PLZ (15mg/kg in bacteriostatic water) prior to formalin testing. PLZ was obtained from Sigma-Aldrich.

PEH Groups (n= 11M, 11F): Animals received a single i.p. injection of PEH (30mg/kg in autoclaved PBS, made from a stock in DMSO) prior to formalin testing. PEH was obtained from the laboratory of Dr. Glen Baker (Department of Psychiatry, University of Alberta, Edmonton, AB, Canada, T6G 2B7).

1.1.4 Rotarod Assay

To assess gross locomotor ability and to ensure that drug treatments did not interfere with locomotion, the accelerating rotarod assay was used. Mice were first trained on a 12-rpm fixed speed rotarod until they could stay on for 3 minutes. On the day of testing, after pre-treatment and formalin testing, mice underwent an accelerating version of the test. For this test, mice start on the rotarod at a rate of 4-rpm that accelerates to 40-rpm over the next minute. Time spent on the accelerating rotarod was averaged across three trials.

1.1.5 Experiment Two: Effect of Estrous Cycle Stage

Experiments were conducted to determine possible effects of the estrous cycle and subsequent hormonal changes on nociceptive behavior or drug effects. Mice were pre-treated with either a drug or saline and then underwent formalin testing. The groups were tested VEH (n=21), PLZ (n=12), PEH (n=12), and N^2-acetylphenelzine (N^2-AcPLZ; n=18). Vaginal lavage samples were taken on the day of formalin testing and stained with crystal violet to determine stage of cycle

during testing. Vaginal lavage, crystal violet staining, and vaginal cytology procedures were similar to those in Maclean et al. (McLean et al., 2012). Stages were distinguished by identifying the predominance of three different cell types: nucleated epithelial cells, cornified squamous cells, and leukocytes. Proestrus is dominated by nucleated epithelial cells**,** estrus by cornified squamous epithelial cells, diestrus is overwhelmingly dominated by leukocytes and metestrus is a mixture of cells. As the mice were free cycling, mice were divided into two groups for the analysis: those in pro-estrus and those in non-proestrus, similar to groups described by Rahn et al.(Rahn et al., 2014).

1.1.6 Experiment Three: The Effect of N^2-AcPLZ on Nociceptive Behaviour

To determine the effect of altering 5-HT and NA levels without changing GABA levels, an acetylated version of PLZ was used. N^2-AcPLZ is an MAO inhibitor and acts to increase levels of 5-HT and NA but not GABA (McKenna et al., 1991). Animals were pre-treated with N^2-AcPLZ (39.82mg/kg in bacteriostatic water, n=10M, 10F) or vehicle (bacteriostatic water, n= 10M, 11F) prior to formalin. N^2-AcPLZ was obtained from the laboratory of Dr. Glen Baker (Department of Psychiatry, University of Alberta, Edmonton, AB, Canada, T6G 2B7).

1.1.7 Experiments Four & Five: Effect of Gonadectomy on Mice Pre-treated with N^2-AcPLZ

Ovariectomized (OVX), castrated (Cast), and sham female/male mice were tested after pre-treatment with N^2-AcPLZ or vehicle. Surgical procedures were performed by Charles River Canada. For ovariectomies, a small incision was made in the dorsal side of the mice and the ovaries were suctioned out. Wounds were closed with clips and removed 7 days after surgery. Sham mice underwent the same surgical procedure without removal of the ovaries. For castration, testes were bilaterally removed after a small incision was made on the ventral side of the mice. Wounds were also close with clips and removed 7 days after surgery. All mice were anesthetized with ketamine/xylazine during surgery and post-operative recovery was mediated with buprenorphine (an opioid) and carprofen (an anti-inflammatory). Mice were shipped after clip removal and allowed to acclimatize to the new environment for 2 weeks prior to testing. On the day of formalin testing, mice received either an i.p. injection of vehicle (bacteriostatic water, Sham Females n=10, Sham Males n=10, OVX n=10, Cast n=10) or N^2-AcPLZ (39.82mg/kg in bacteriostatic water; Sham Females n=10, Sham Males n=10 , OVX n=10, Cast n=10) prior to

formalin injection as previously described. An estrus sample was also obtained from female mice prior to tissue removal to later ensure that ovariectomized mice were no longer cycling. Estrus cycle stages were determined as previously described. Non-cycling mice were divided into two categories based on vaginal lavage samples: anestrus, described by a mixture of unidentifiable cell types, and prolonged diestrus, characterized by a large amounts of leukocytes, typical of periods of low estrogen and progesterone (Aloisi et al., 2004; Hayashi et al., 2013; Chen et al., 2014). Castrated and Sham surgery male mice were not tested until a minimum of 14 days after surgery to ensure that all circulating testosterone had diminished (Xu et al., 1999; Lin et al., 2010; Boerboom et al., 2015).

1.1.8 Experiment Six: Pharmacological Antagonism of N^2-AcPLZ-mediated Anti-nociception in Male Mice.

Different pharmacological agents were used to determine if the analgesic effects of N^2-AcPLZ in male mice could be reversed. All antagonists used have previously been shown to not alter formalin behaviour when compared to vehicle treatment alone (Rocha-González et al., 2005; Roh et al., 2006; Kang et al., 2011; Cervantes-Durán et al., 2013; Muñoz-Islas et al., 2014). Male mice were pre-treated with N^2-AcPLZ or vehicle (bacteriostatic water). Twenty minutes prior to formalin testing, mice received an intrathecal (i.t.) injection of either vehicle or drug (see drugs used below). I.t. injections were carried out under anesthesia with isoflurane at the lower lumbar level. A volume of 10µl was administered with a 30-gage needle. Puncture of the dura was confirmed by a tail flick at the time of injection and later through visualization of the dye injected with the drug in the spinal column near the site of injection. Once the mice had recovered from the isoflurane induction, formalin testing occurred as previously described. Mice were also tested on the accelerated rotarod in a single trial (mice start on the rotarod at a rate of 4-rpm that accelerates to 40-rpm over the next minute. Time spent was averaged across three trials) to ensure that locomotor ability was not impaired by the i.t. injections. After testing, mice were euthanized with euthansol and the spinal column was opened at the lumbar level to verify proper intrathecal localization of the injection (visualized by the presence of blue dye in the spinal tissue).

1.1.9 Experimental Groups and Drugs Used:

Vehicle - Vehicle (n=7): mice were given an i.p. injection of bacteriostatic water and an i.t. injection of 1% DMSO (10µl) prior to formalin testing.

N^2-AcPLZ - Vehicle (n=8): mice were given an i.p. injection of N^2-AcPLZ (39.82mg/kg in bacteriostatic water) and an i.t. dose of 1% DMSO (10 µl volume) prior to formalin testing.

N^2-AcPLZ - Idazoxan hydrochloride (n=6): Idazoxan hydrochloride is an alpha 2a-adrenoreceptor antagonist. Mice were given an i.p. injection of N^2-AcPLZ (39.82mg/kg in bacteriostatic water) and an i.t. dose of idazoxan hydrocholide (2mg/kg in bacteriostatic water) prior to formalin testing. Idazoxan hydrocholide was purchased from Tocris BioSciences, Cedarlane Labs, Burlington, ON.

N^2-AcPLZ - SB-699551 (n=6): SB-699551 is a 5HT-5A receptor antagonist. Mice were given an i.p. injection of N^2-AcPLZ (39.82mg/kg in bacteriostatic water) and an i.t. injection of SB-699551 (3mg/kg in DMSO) prior to testing. SB-699551 was purchased from Tocris BioSciences, Cedarlane Labs, Burlington, ON.

N^2-AcPLZ - WAY - 100635 (n=8): WAY-10063 is a 5HT-1A/B/D receptor antagonist, with a strong affinity for the 5HT-1a receptor. Mice were given an i.p. injection of N^2-AcPLZ (39.82mg/kg in bacteriostatic water) and an i.t. injection of WAY-100635 (1.5µg in 10µl 0.9% saline) prior to testing. WAY-100635 was purchased from Tocris BioSciences, Cedarlane Labs, Burlington, ON.

1.1.10 High Performance Liquid Chromatography

Mice from all experiments were euthanized via a euthansol injection and transcardiac perfusion with saline after testing. Whole spinal cords were dissected fresh and flash frozen in liquid nitrogen. The tissue was then used to prepare samples for high performance liquid chromatography (HPLC) analysis to determine levels of amino acids and biogenic amines. Procedures outlined by Musgrave et al. [49] were used in the present study with minor modifications [49]. Tissue used for amino acid analysis was homogenized in 5 volumes of ice cold water. An aliquot was added to 4 volumes ice cold methanol and left on ice for 10 min and then centrifuged (12000*g* for 4 min). The supernatant was then diluted using distilled water to a final 120-fold dilution. Portions of the resulting supernatant were then reacted with o-pthaldialdehyde (OPA) and N-iso-butyryl-L-cysteine (IBC) dissolved in a borate buffer and the

resulting derivatives were used for analysis with a fluorescence detector set at an excitation wavelength of 344 nm and an emission wavelength of 433m. Calibration curves for amino acids were prepared from authentic amino acid samples and generated for each individual run of samples.

For separation and analysis of biogenic amines, procedures used by Musgrave et al. were used with minor modifications (Musgrave et al., 2011a). Aliquots of tissues homogenized in 5 volumes of water were combined with 1/10th the volume of ice-cold 1M $HClO_4$ containing ascorbic acid (500μm) and EDTA (100 mg%). Homogenates were vortexed and then centrifuged at 12000*g* for 4 minutes. The resulting supernatants were then used for analysis by HPLC using electrochemical detection. The applied potential for electrochemical detection was 0.65 V. Calibration curves were constructed for each run.

All water used was distilled and purified by reverse osmosis using the Milli-Q filtration system from Millipore. Methanol, tetrahydrofuran, and acetonitrile were HPLC grade from Fisher Scientific. All solvents were filtered using Millipore nylon membranes (0.2μm pore size). OPA and ascorbic acid were from Sigma Aldrich, IBC from Novachem and sodium borate from Fisher Scientific.

1.1.11 Statistical Analysis

Analyses were conducted using SigmaPlot software version 12.0. Total time spent engaging in nociceptive behaviour in the first (phase 1) and second (phase 2) phases were analyzed using one-way ANOVAs to examine drug effects, followed by Bonferroni *post hoc* tests as needed. Independent Student's two tailed t-tests were used to test two treatment group experiments' phase 1 and phase 2 times. Non-parametric tests (ANOVA on ranks) were used where assumptions were not met. A percent reduction in pain score was also calculated for second phase formalin scores in experimental groups. This was calculated using the following equation: ((individual time (s)/total vehicle time(s)) -1) x100. Differences in estrous cycles were determined using Independent Student's two-tailed t-tests. HPLC results were analyzed using a two way ANOVA to examine a possible interaction between sex and drug, with Bonferroni *post hoc* t-tests as needed for experiment one. One-way ANOVAs were used to analyze results from rotarod testing with *post hoc* analysis as needed. In all cases, results were considered statistically significant if $p<0.05$.

1.2 Results

1.2.1 Impact of PLZ and PEH Treatment on Nociceptive Behaviour in the Formalin Test

To explore the effect of altering specific CNS neurotransmitter levels on nociceptive behaviour in male and female mice, we used the formalin test after pre-treatment with PLZ, PEH, or vehicle.

In male mice, the overall time spent engaging in nociceptive behaviours was analyzed after being divided into phase 1 (first 10 minutes of observation period) and phase 2 (remaining 20 minutes of observation period). A one-way between-subjects ANOVA conducted to compare the effect of treatment on time spent engaging in nociceptive behaviour during phase 1 was significant at the $p<0.05$ level for the treatment conditions ($F_{3,35}$=5.644, P=0.003; Fig. 1A). *Post hoc* analysis using a Bonferroni t-test revealed that the environmental control group spent significantly less time engaging in nociceptive behaviour compared to all other groups (vs VEH t= 3.599, P=0.006, vs PLZ t=3.811, P=0.003, vs PEH t=3.567, P=0.006; Fig. 1A). A second one-way between-subjects ANOVA conducted to compare the effect of treatment on time spent engaging in nociceptive behaviour in phase 2 was significant for the treatment conditions ($F_{3,35}$=8.094, P<0.001). Bonferroni t-test *post hoc* tests showed that PLZ- (t=3.401, P=0.010) and PEH- (t=2.979, P=0.031) treated mice spent significantly less time engaging in nociceptive behaviour compared to vehicle treated mice (Fig. 1B). Male mice treated with vehicle also spent significantly more time engaging in nociceptive behaviour compared to environmental controls (t=4.586, P<0.001; Fig. 1B). It is interesting that the environmental group and the two drug-treated groups did not differ significantly, indicating that drug treatment lowered nociceptive attending behaviours to a level comparable to a non-formalin treated animal. Overall, nociceptive behaviour was reduced by 54% with PLZ treatment and 50% with PEH treatment compared to vehicle (VEH) treated male mice (Fig. 1B). No differences were found between treatment groups for performance in the accelerating rotarod assay, indicating the drug treatments did not interfere with motor function in treated male mice (Fig. 1C).

In female mice, no significant differences in the duration of responses during phase 1 with regard to treatment were found (Fig. 1D). However, female mice in the environmental control group spent less time engaging in nociceptive behaviour compared to vehicle-treated

mice (Kruskal-Wallis H test H_3=8.366, P=0.039, followed by *post hoc* analysis with Dunn's Method, Q=2.809, P<0.05; Fig. 1D).

Analysis of the second phase of formalin-evoked nociceptive behaviours in female mice using a Kruskal-Wallis H test showed that there was a significant difference in time spent engaging in nociceptive behaviour between drug groups, (H_3=17.286, P<0.001; Fig. 1E). *Post hoc* tests (Dunn's Method) revealed that only mice treated with PEH spend significantly less time engaging in nociceptive behaviour compared to vehicle (Q=3.928, P<0.05; Fig. 1E), and that only vehicle-treated mice spend significantly more time engaging in nociceptive behaviour compared to environmental controls (Q=3.928, P<0.05; Fig. 1E). Treatment with PLZ only reduced nociceptive behaviour by 40%, but treatment with PEH caused a 62% reduction in nociceptive behaviour in female mice. Finally, analysis of the performance in the accelerating rotarod assay in female mice revealed no significant differences between treatment groups (Fig. 1F).

1.2.2 Effect of Estrus Stage on Formalin Behaviour

We next assessed whether the estrous cycle had any influence on the response to treatment. We therefore conducted a second experiment to assess the estrous phase on the day of formalin testing in female mice pretreated with PLZ, PEH, or VEH using crystal violet staining (Fig 2A). Stages were determined on the basis of the predominance of different cell types (see the Methods section, and Fig 2A). We saw a similar pattern of behavior in response to formalin testing in this experiment. No significant differences were seen in phase 1 behavior (Fig 2Bi). During phase 2, only mice treated with PEH showed a significant reduction in nociceptive behavior ($F_{2,32}$ = 4.781, P = .015, Bonferroni *post hoc* for PEH t = 2.871, P = .014, for PLZ t = 2.066, P = .094; Fig 2Bii). In this experiment, we observed a 34% reduction in nociceptive behavior with PLZ treatment and that treatment with PEH resulted in a 43% reduction (Fig 2B). Mice were then classified as being in proestrus (the stage with the greatest hormonal changes) or in non-proestrus stages for analysis. This was done for phase 1 and phase 2 of the formalin response testing to determine if estrous stage affected the time spent engaging in nociceptive behaviors during the assay. No differences were found between groups for either phase 1 or phase 2 in VEH-, PLZ-, or PEH-treated mice (P > .05, 2-tailed t-tests; Figs 2C–2E).

1.2.3 Effect of PLZ and PEH on the Levels of Major Spinal Cord Neurotransmitters

To quantify the effect of treatment on major spinal cord neurotransmitters, HPLC was used to determine if PLZ or PEH altered the levels of 5-HT, NA, and/or GABA in the spinal cord and if these changes differed between sexes.

GABA: The influence of sex and treatment on spinal cord GABA levels was determined using a two-way between-subjects ANOVA (Fig. 3A). A significant interaction was found for sex and drug treatment ($F_{4,39}$=6.469, P<0.001). This was followed by *post hoc* analyses (Bonferroni t-tests). In male mice, PEH pre-treatment significantly elevated levels of GABA in the spinal cord compared to naïve males (t=3.546, P=0.010), male mice in the environmental control condition (t= 3.077, P=0.038), and vehicle-treated male mice (t= 3.224, P=0.026; Fig. 3A). A trend toward significant increases in the PLZ-treated group was also seen (Fig. 3A).

In female mice, GABA levels were increased significantly more in PEH pre-treated groups compared to naïve females (t= 8.321, P<0.001), female mice in the environmental control condition (t=7.819, P<0.001), and vehicle pre-treated female mice (t=7.980, P<0.001; Fig. 3A). PLZ-treated female mice also had more GABA compared to naïve females (t= 6.875, P<0.001), female mice in the environmental control condition (t=6.308, P<0.001), and vehicle-treated female mice (t=6.534, P<0.001; Fig. 3A).

Significant sex differences were also observed for changes in spinal GABA levels in response to treatment. Both PLZ- and PEH- treated females had significantly higher GABA levels compared to males treated with PLZ (t= 4.822, P<0.001) or PEH (t= 4.469, P<0.001; Fig. 3A).

5-HT: Analysis of spinal cord 5-HT levels also revealed treatment and sex differences. An interaction between sex and drug was found using a two-way between-subjects ANOVA ($F_{4,39}$=4.335, P=0.005; Fig. 3B). In males, PLZ-treated mice had significantly more 5-HT compared to naïve males (t=9.534, P<0.001), male mice in the environmental control condition (t= 11.737, P<0.001), vehicle treated male mice (t= 8.449, P<0.001), and PEH treated male mice (t=8.376, P<0.001; Fig. 3B). Male mice treated with PEH also had more 5-HT compared to male mice in the environmental control condition (t=3.361, P=0.017) and male mice pre-treated with vehicle (t=3.288, P=0.021; Fig. 3B). Female mice treated with PLZ also had significantly more 5-HT compared to naïve females (t=5.243, P<0.001), female mice in the environmental control

condition (t= 7.618, P<0.001), vehicle-treated females (t= 3.501, P=0.012), and PEH-treated females (t=5.222, P<0.001; Fig. 3B).

Sex differences in the changes of 5-HT levels with treatment were also observed. Male mice treated with PLZ have a significantly greater surge in 5-HT levels compared to PLZ-treated females (t= 4.586, P<0.001; Fig. 3B).

NA: Changes in spinal NA levels were also examined. No significant interaction was found for drug and sex, but main effects for sex ($F_{1,40}$=11.486, P=0.002) and drug ($F_{4,40}$=17.766, P<0.001) were found (Fig. 3C). *Post hoc* analyses for the main effect of drug revealed that all PLZ-treated mice had significantly more NA compared to naïve mice of both sexes (t= 7.911, P<0.001) and to environmental controls (t= 5.674, P<0.001) and vehicle-treated (t= 6.107, P<0.001) and PEH-treated mice (t=4.400, P<0.001; Fig. 3C). PEH-treated mice also had more NA compared to naïve mice (t= 3.643, P=0.008; Fig. 3C). Although these levels were elevated compared to naïve controls, overall the levels of NA in PEH-treated mice were comparable to mice in the environmental condition that received saline injection into the hind paw. This suggests that the increases in NA in PEH, vehicle, and environmental controls were due to factors such as the pain from formalin injection or the needle itself, as opposed to systemic treatment with PEH. Analysis for the main effect of sex revealed that males had significantly more NA compared to females overall (t= 3.389, P=0.002, Fig. 3C).

1.2.4 Effect of N^2-AcPLZ on Formalin Nociceptive Attending Behaviours

At the spinal level we found that female mice exhibit much larger increases in GABA while males have significantly greater changes in 5-HT and NA levels in response to treatment. Given the significant sex differences in the responses to treatment, we next wanted to test the effects of a drug that only elevates 5-HT and NA. N^2-AcPLZ is an MAO inhibitor but, unlike PLZ, does not interfere with GABA-transaminase. Treatment with N^2-AcPLZ leads to increases in 5-HT and NA but does not significantly elevate GABA levels (McKenna et al., 1991).

Formalin experiments in males revealed no significant difference in formalin behaviour for phase 1 (Fig. 4A). In phase 2, N^2-AcPLZ pre-treatment significantly lowered the time spent engaging in nociceptive behaviour in male mice (t= 2.535, P=0.021; Fig. 4B). There was a 56% reduction in formalin-induced nociceptive behaviour compared to controls.

In females, a two-tailed t-tests for phase 1 and phase 2 nociceptive behaviours showed no significant differences between treatment groups (Fig. 4C, D). There was only a 24% reduction in nociceptive behaviour with N^2-AcPLZ treatment. These results indicate that N^2-AcPLZ pre-treatment does not help attenuate nociceptive behaviours in female mice.

Estrus cycle monitoring was also carried out for female mice pre-treated with N^2-AcPLZ. As found previously, no differences were seen between mice in proestus versus non-proestrus for the time spent engaging in nociceptive behaviour for either phase 1 or phase 2 of formalin testing (Fig. 4E).

1.2.5 Effect of N^2-AcPLZ on Spinal Cord Neurotransmitters

We next carried out HPLC analysis on spinal cord tissue samples from male and female mice pre-treated with N^2-AcPLZ. Similar to the analyses in experiment 1, 5-HT and NA levels were analyzed using two-way between subjects ANOVAs to determine the influence of drug treatment and sex on neurotransmitter levels.

A significant main effect of drug was found for 5-HT spinal cord levels ($F_{1,16}$=95.310, P<0.001) and pre-treatment with N^2-AcPLZ was found to significantly increase 5-HT levels overall (t=9.763, P<0.001; Fig. 5A). Similarly, a main effect of drug was found for the spinal levels of NA ($F_{1,16}$=23.492, P<0.001) and pre-treatment with N^2-AcPLZ was found to significantly increase NA levels overall (t=4.847, P<0.001; Fig. 5B). These results indicate that treating male or female mice with N^2-AcPLZ significantly elevates 5-HT and NA levels in the spinal cord. However, only male mice respond with an attenuation of nociceptive behaviours in response to formalin stimulation.

1.2.6 Effect of Gonadectomy on Responsiveness to N^2-AcPLZ

To determine if removal of female gonadal hormones (estrogen and progesterone) would alter the ability of N^2-AcPLZ to attenuate nociceptive behaviour in female mice, we next assessed the responses of ovariectomized (OVX) mice after pre-treatment with N^2-AcPLZ in the formalin test. Vaginal lavage samples were taken on the day of testing and stained using crystal violet to determine if a lack of female gonadal hormones in OVX mice could be identified by lack of regular cycling stages (Fig. 6A,B). Stages were determined based on the predominance of different cell types (see Methods, Fig. 6A,B). Mice in the sham surgery condition (sham-OVX)

were free cycling normally. This is demonstrated by the fact that sham-OVX mice can be found to be in each of the stages of the estrus cycle (Fig. 6C). Vaginal lavage sample cytology from OVX mice did not match normal cycling estrus stages and were thus described as anestrus (no identifiable cell types) or prolonged diestrus (only large amounts of leukocytes identifiable; Fig. 6B,C).

Time spent engaging in nociceptive behaviours in phase 1 and phase 2 was analyzed using one way between subject ANOVAs. No significant differences were found between groups (Fig. 6E,F). Only a respective 37% and 23% reduction in nociceptive behaviour was seen in N^2-AcPLZ-treated Sham and OVX mice. The absence of any differences between OVX and sham-OVX mice in response to N^2-AcPLZ suggests that the overall lack of an anti-nociceptive response to N^2-AcPLZ in female mice is not related to some inhibitory influence mediated by the presence of female gonadal hormones.

Since removal of female gonadal hormones (estrogen and progesterone) did not alter the ability of N^2-AcPLZ to attenuate nociceptive behaviour in female mice, we next assessed whether testosterone had an effect on the responses to N^2-AcPLZ in male mice using castrated (Cast) males. Time spent engaging in nociceptive behaviours in phase 1 and phase 2 was analyzed using one way between subject ANOVAs. No significant differences were found between groups in phase 1 (Fig. 7Ai). Time spent engaging in nociceptive behaviour in phase 2 was significantly reduced ($F_{(3,33)}$= 12.301, P<0.001; Fig. 7Aii). *Post hoc* analysis showed that N^2-AcPLZ significantly reduced nociceptive behaviours in both sham (vs. Sham Veh: t=3.246, P=0.016, vs. Cast Veh: t=3.424, P=0.01) and castrated mice (vs. Sham Veh: t=4.814, P<0.001, vs. Cast Veh: t=5.513, P<0.001) when compared to both control groups (Fig. 7Aii). Sham surgery and castrated mice treated with N^2-AcPLZ (N2) demonstrated a 48% and 72% reduction in nociceptive behaviour respectively. The significant reduction in nociceptive behavior in both sham and castrated males, along with the data from ovariectomized mice suggests that gonadal hormones are not mediating the sex differences seen in the nociceptive behaviors.

1.2.7 Determining the Mechanism of N^2-AcPLZ-mediated Anti-nociception in Male Mice

We next wanted to determine how N^2-AcPLZ mediates its anti-nociceptive effect in male mice. We used different pharmacological antagonists (Idazoxan hydrochloride: alpha 2a-adrenoreceptor antagonist; SB-699551: 5-HT 5A antagonist; WAY-100635: 5-HT 1A/B/D

antagonist) to antagonize receptors known to be involved in analgesia and assess if these could reverse the anti-nociceptive effects of N^2-AcPLZ in male mice.

A one-way between-subjects ANOVA for time spent engaging in nociceptive behaviour during phase 1 was found to be significant ($F_{4,30}$=3.966, P=0.011; Fig. 8A). *Post hoc* analysis using a Dunnett's t-test revealed that mice treated with Idazoxan hydrochloride spent significantly less time engaging in nociceptive behaviour compared to VEH/VEH mice during the first phase of the formalin response (VEH/VEH vs N2/Idaz t= 3.549, P=0.005; Fig. 8A). A Kruskal-Wallis H test conducted on time spent engaging in nociceptive behaviour in phase 2 was significant (H_4=17.347, P=0.002; Fig. 8B). Dunn's Method showed that N2/VEH- (Q=2.579, P<0.05), N2/Idaz- (Q=3.174, P<0.05), and N2/SB- (Q=3.393, P<0.05) treated mice spent significantly less time engaging in nociceptive attending behaviour compared to VEH/VEH mice (Fig.8B). Interestingly, there was no significant difference in the time spent engaging in nociceptive attending behaviours in the second phase of the formalin response between VEH/VEH and N2/WAY treated mice. Thus, WAY-100635 was the only antagonist that prevented the anti-nociceptive effect of N^2-AcPLZ in male mice. This suggests that the analgesic properties of N^2-AcPLZ in male mice are mediated through the 5HT-1 class of receptors.

1.3. Discussion

Pre-treatment with the MAO-inhibitor PLZ or its metabolite PEH prior to formalin testing was used to assess the effects of elevating GABA, 5-HT, and NA (PLZ) or GABA alone (PEH) on nociceptive behaviours between the sexes. There were no differences between any of the treatment groups in phase 1 of the formalin response for either male or female mice. PLZ and PEH were, however, effective at attenuating second phase behaviours in a sex-specific manner.

In initial behavioural experiments, we found that nociceptive behaviour was significantly reduced in both sexes with PEH pre-treatment. With PLZ pre-treatment, a significant reduction in nociceptive behaviour was only seen in male mice, with a trend towards decreased nociceptive behaviours in female mice. Given the behavioural differences seen between male and female responses to these treatments, we monitored the estrous cycle in female mice to determine if daily fluctuations in gonadal hormones impacted nociceptive responses to formalin. The second experiment had a similar pattern of results as the first experiment, with only PEH treatment causing a significant reduction in nociceptive behaviours in females and a trend towards a

decrease in nociceptive behaviours with PLZ pre-treatment. Overall, we did not observe any differences in the nociceptive responses of female mice across different time points in the estrous cycle. These results are in line with earlier studies that concluded that the day-to-day hormone fluctuations associated with the estrous cycle do not have a significant impact on nociception in the formalin model (Wichmann et al., 1996; Kuba et al., 2006; Kim et al., 2015).

We then explored changes in spinal neurotransmitter levels using HPLC. We found that drug-treated female mice had significantly larger increases in endogenous levels of GABA compared to drug-treated male mice. This increase may account for the significant reduction in nociceptive behaviour with PEH pre-treatment and the slight reduction in nociceptive behaviour with PLZ. Given the similar increase in GABA in females with both PLZ and PEH treatment, it is surprising that both of these drugs were not equally effective in reducing nociceptive behaviour. This may suggest that the increases levels of 5-HT/NA with PLZ may possibly have presently unknown actions in the female spinal cord to prevent the anti-nociceptive properties of PLZ.

We also found that elevations in 5-HT and NA levels with PLZ treatment were significantly greater in male mice compared to females and a significant reduction in nociceptive behaviour was seen in male mice. Given that there was only a slight decrease in nociceptive behaviour in female mice with PLZ treatment, and a smaller increase in 5-HT/NA in females treated with PLZ, there may be underlying differences in how males and females can use 5-HT/NA to manage nociception. On the basis of this idea, we conducted an experiment to test the hypothesis that females would be unresponsive to a treatment that only elevated 5-HT and NA. Using the compound N^2-AcPLZ, which only elevates 5-HT and NA, we found that male mice had a significant attenuation of second phase formalin behaviour compared to controls. As predicted, female mice treated with N^2-AcPLZ did not differ in their responses compared to vehicle-treated controls. Interestingly, 5-HT and NA levels were equally elevated in the spinal cords of both sexes with this treatment. The equal increase of spinal 5HT/NA in both sexes, yet the different behavioural results suggest that underlying sex-specific pathways may be in place to modulate nociception differently in males and females. Male anti-nociception in this context appears to be regulated by 5-HT and NA, while female anti-nociception does not. Similar sex-specific anti-nociceptive mechanisms have been observed in relation to opioid analgesia, and studies of toll-like receptor 4-mediated analgesia have also revealed distinct nociceptive

mechanisms in males and females (Craft, n.d.; Mogil et al., 2003; Berkley et al., 2006; Fillingim et al., 2009; Sorge et al., 2011; Mogil, 2012).

Although our findings indicate that 5HT, NA, and GABA are important for the regulation of nociception, it is important to acknowledge that other neurotransmitter systems have a role in these processes. Studies indicate that dopamine has a role in pain processing, especially in the transition from acute to chronic pain (Yang et al., 2005; Chang et al., 2014; Kim et al., 2015; Sagheddu et al., 2015). Other studies have shown that drugs that alter dopamine levels in the CNS have beneficial effects on pain (Cobacho et al., 2014; Hache et al., 2015). Since PLZ and N^2-AcPLZ both act by inhibiting the MAO enzyme, they both elevate dopamine levels in the CNS (McKenna et al., 1991). Although the role of dopamine was not explored in the present study, it is important to acknowledge that dopamine may contribute to the attenuation of nociception seen in males and the partial reduction in pain in females, and therefore may possibly relate to the sex differences we have observed with these compounds.

Previous work has suggested that global gonadal hormone levels can affect pain regulation at the spinal level (Aloisi et al., 2004; Loyd & Murphy, 2014; McCarson & Enna, 2014; Vincler et al.,2001). Estrogen can interact with both GABA- and NA-mediated nociception. Estrogen has also been shown to gate GABAergic tone. High levels of estrogen result in a decrease in GABAergic tone, leading to greater neuronal excitability (Tashiro et al., 2014). The work by Tashiro et al. (2014) suggests that increasing GABAergic tone in females may be beneficial for anti-nociception (Tashiro et al., 2014). This may explain why PEH was particularly effective in female mice in the current set of experiments (Nag and Mokha, 2014; Besson et al., 2015). In addition, it has been observed that activation of membrane estrogen receptors through estradiol can attenuate the analgesic effects of α2-adrenoreceptor agonists in females (Nag and Mokha, 2014). These studies suggest that treatments that increase NA (such as PLZ and N^2-AcPLZ used in the present study), may not promote anti-nociception in female mice due to the inhibitory effects of estrogen.

To further explore the role of female gonadal hormones on formalin induced nociceptive behaviour, we used ovariectomized female mice in the formalin assay. We postulated that the removal of estrogen and progesterone might create a more permissive environment for females to use 5-HT and NA for the attenuation of nociceptive behaviours. Removing female gonadal hormones did not, however, influence the response to N^2-AcPLZ pre-treatment. OVX-female

mice remained unresponsive to N^2-AcPLZ pre-treatment. Since removal of female gonadal hormones did not alter the female response to N^2-AcPLZ, we tested the hypothesis that testosterone may be mediating the effects of N^2-AcPLZ. We postulated that the presence of sufficient testosterone levels may be required for receptors to become engaged and reduce nociceptive attending behaviours, which has been reported in other papers (Mogil et al., 2003; Sternberg et al., 2004; Juni et al., 2010; Sorge et al., 2011; Mogil, 2012). This would also explain why, although we find similar increases in 5HT and NA levels in both sexes with N^2-AcPLZ pre-treatment, anti-nociception is observed only in male mice. To our surprise, castration of male mice had the opposite effect. We found that castrated male mice treated with N^2-AcPLZ had reduced nociceptive behaviours in phase 2 of the formalin response. These results, combined with those of the ovariectomy experiment suggest that the sex differences observed in these studies are not due to circulating gonadal hormones. It is possible however, that gonadal hormones exert their effects during development to establish the system (Cicero et al., 2002; Loyd and Murphy, 2006). Alternatively, there may exist underlying sex chromosome complement differences outside of the gonads (Gioiosa et al., 2008a, 2008b).

To further explore the mechanism underlying the anti-nociception with N^2-AcPLZ in male mice, we went on to conduct studies aimed at determining which receptor/receptors N^2-AcPLZ might be acting through to mediate its anti-nociceptive effects in males. 5HT-5A, 5HT-1A, and α-2A adrenoreceptors are all known to be involved in spinal mechanisms of anti-nociception (Andurkar et al., 2012; Zhao et al., 2012, 2014; Bhalla et al., 2013; Cervantes-Durán et al., 2013). Blocking 5HT-1A receptors with WAY-100635 was the only treatment that effectively reversed N^2-AcPLZ-mediated anti-nociception. This suggests that elevated 5-HT in response to N^2-AcPLZ acts through the 5HT-1A receptor in the spinal cord to mediate anti-nociception in male mice after formalin stimulation. Sexual dimorphisms in the 5-HT1A receptor have been explored in various contexts. A study of prenatal stress and pain in male and female infant rats have shown that the 5-HT1A mediated functional maturity of descending serotonergic inhibitory system appears earlier in males than in females (Butkevich et al., 2011). This observation may explain why gonadectomies did not have an effect on N^2-AcPLZ mediated anti-nociception in the present study. Furthermore, a study of sex differences in 5-HT1A binding affinity in a healthy human population showed a small mean reduction of binding potential in

women relative to men in the prefrontal and occipital cortex, suggesting possible innate receptor expression differences between the sexes (Moses-Kolko et al., 2011).

1.4 Conclusions

In summary, our results indicate that different underlying mechanisms in response to drug treatment exist in male and female mice for the attenuation of nociceptive behaviour after formalin stimulation. Using behavioural models and HPLC analysis, we have identified a dichotomy in the neurotransmitter systems involved in regulating nociception between the sexes. Our data demonstrate that males can utilise 5-HT and NA to decrease nociceptive responses when treated with drugs that alter these neurotransmitter systems, while females cannot. Given that the first line drugs for chronic pain increase the functional availability of 5-HT and NA, this needs to be explored further to determine if drug that act to increase these neurotransmitters are the most effective treatment for females with chronic pain (Coderre et al., 1990; Coderre and Melzack, 1992; Xu et al., 1999; Hains et al., 2002; Sommer, 2004b; Pertovaara, 2006; Hughes et al., 2013b). Our additional observation that elevating GABA levels was successful at decreasing nociceptive behaviour in both males and females provides support for the development of drugs that act through GABA to treat persistent pain (McCarson and Enna, 2014; Besson et al., 2015). Unfortunately, many GABAergic drugs can have problematic side effects, such as sedation. Although sedation was not a prominent effect in the present experiment, this is a factor to consider in future clinical studies. Overall, these findings have significant implications for the design of future analgesic therapies, suggesting that males and females may require different drugs to effectively control pain

.

1.5 Figures

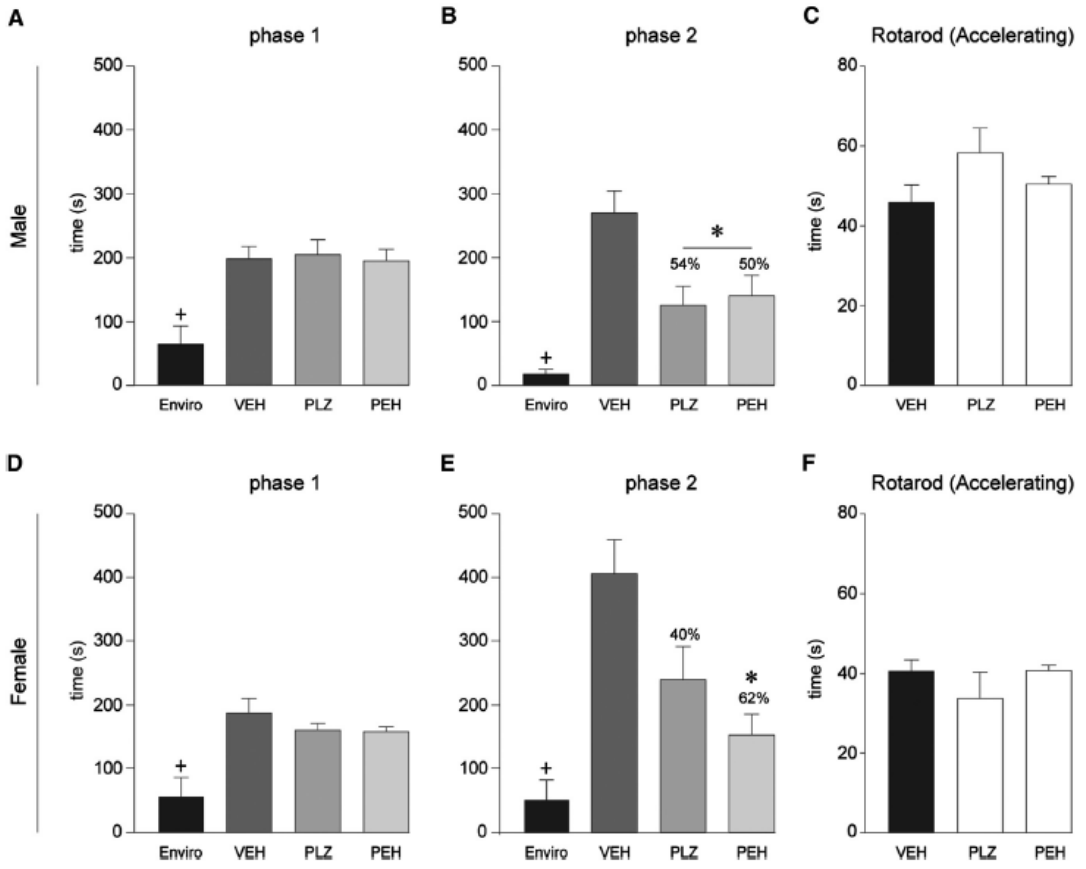

Figure 1.1. *Male and female response to formalin testing*. **A, D.** Nociceptive behaviour was not altered by either PLZ or PEH during phase 1 of formalin testing in either male or female mice. **B**. PLZ and PEH significantly reduced the time spent engaging in nociceptive behaviour during phase 2 of the formalin test in males. Nociceptive behaviour was reduced by 54% with PLZ treatment and 50% with PEH treatment compared to VEH-treated male mice. **C, F.** Rotarod motor testing revealed no differences in motor function after drug treatments in either female or male mice. **E.** Although treatment with PLZ reduced nociceptive behaviour by 40%, only treatment with PEH, a 62% reduction, significantly reduced nociceptive behaviour in female mice. **A, B, D, E.** Environmental control (Enviro; i.p. saline) mice all showed significantly less pain than vehicle groups (+). **One-way ANVOAs, P<0.05. All graphs are mean ±SEM.*

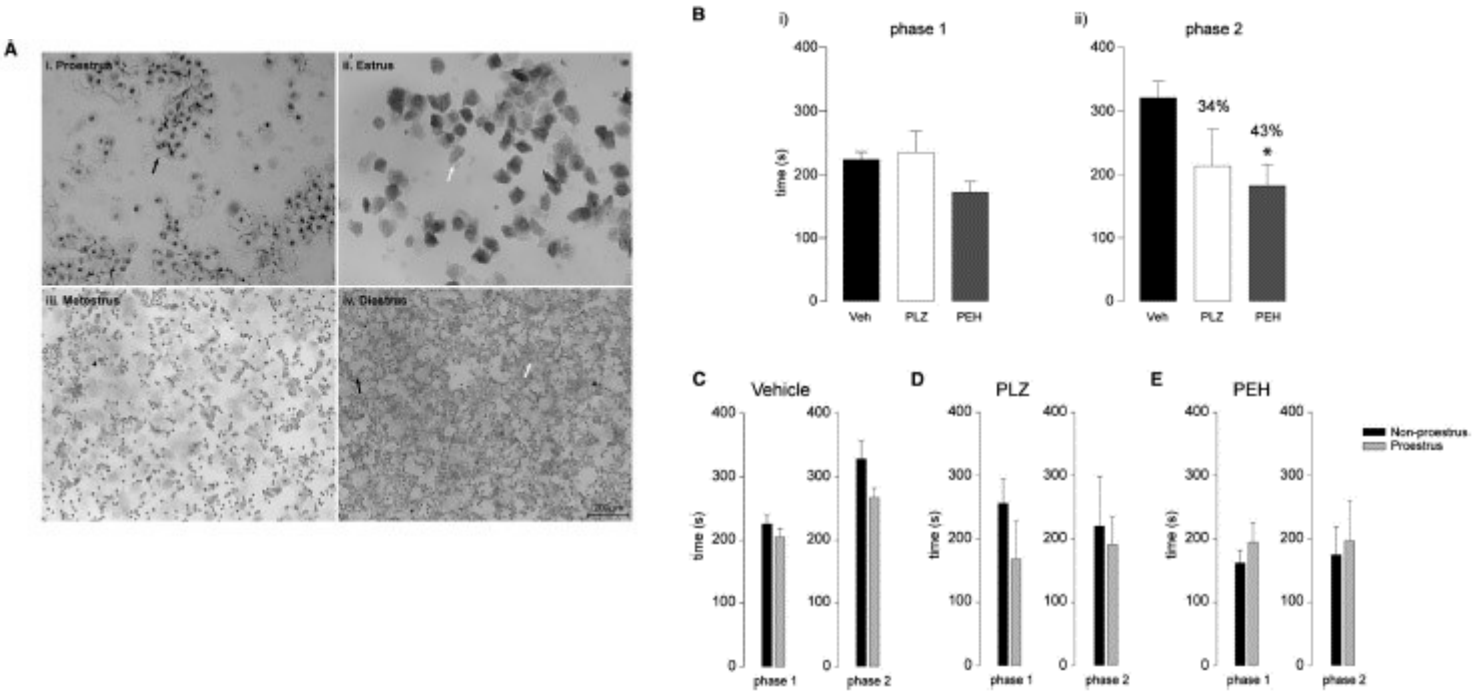

Figure 1.2. *Effect of estrus on female nociceptive behaviour*. **A.** Example pictures from estrus cycle stages. Three main cell types are used to identify estrus cycle stage in vaginal smear samples: nucleated epithelial cells (black arrows), cornified squamous cells (white arrows), and leukocytes (black arrowheads). **Ai**. Proestrus is dominated by nucleated epithelial cells. **Aii.** Estrus is dominated by cornified squamous epithelial cells. **Aiii.** Metestrus has a mixture of cornified squamous epithelial cells and leukocytes though other cells may also be mixed in. **Aiv.** Diestrus is overwhelmingly dominated by leukocytes, though other cells types may be mixed in. **Bi.** No differences were seen in phase 1 behaviour**. Bii.** Phenylethylidenehydrazine (PEH) was able to significantly decrease nociceptive behavior compared with VEH, unlike plZ pretreatment.**, C, D, E.** Time spent engaging in formalin induced nociceptive behaviour in phase 1 and 2 of veh-, PLZ- and PEH- treated animals. No significant differences were found. * *Student's t-tests, P<0.05. All graphs are mean ±SEM.*

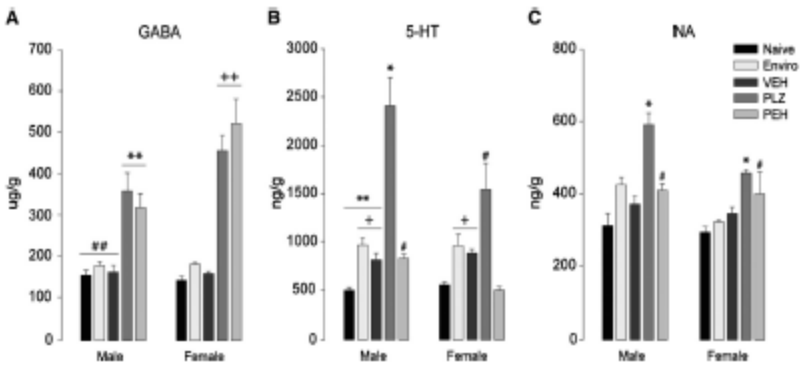

Figure 1.3. *Effect of PLZ, PEH, and sex on levels of major spinal cord neurotransmitters.*
A. Differences in GABA levels using HPLC. ** Male phenelzine (PLZ) and and phenylethylidenehydrazine (PEH) groups had significantly less GABA compared to female counterparts..$^{++}$ Female PLZ and PEH groups had significantly more GABA than the other female group. $^{\#\#}$ PEH males had more GABA compared to naive, VEH-treated (VEH), and environmental control (Enviro; i.p. saline) male mice. **B.** Differences seen in 5-HT levels. * PLZ males had more 5-HT compared to PLZ females.** PLZ males had more 5-HT compared to all other male groups. $^{\#,+}$ There was also a significant difference between PEH, and Enviro males and VEH males, with VEH groups having less 5-HT in both cases. $^{\#}$ PLZ females had higher levels of 5-HT compared to all other female groups..$^{+}$ Enviro females also had more 5-HT compared to VEH females.**C.** Differences in NA levels. *Males had higher levels of NA in general compared to females. PLZ groups had more NA compared to any other group. $^{\#}$ PEH groups also had more NA compared to naïve. *Two-way ANOVAs, P<0.05. All graphs are mean ±SEM.*

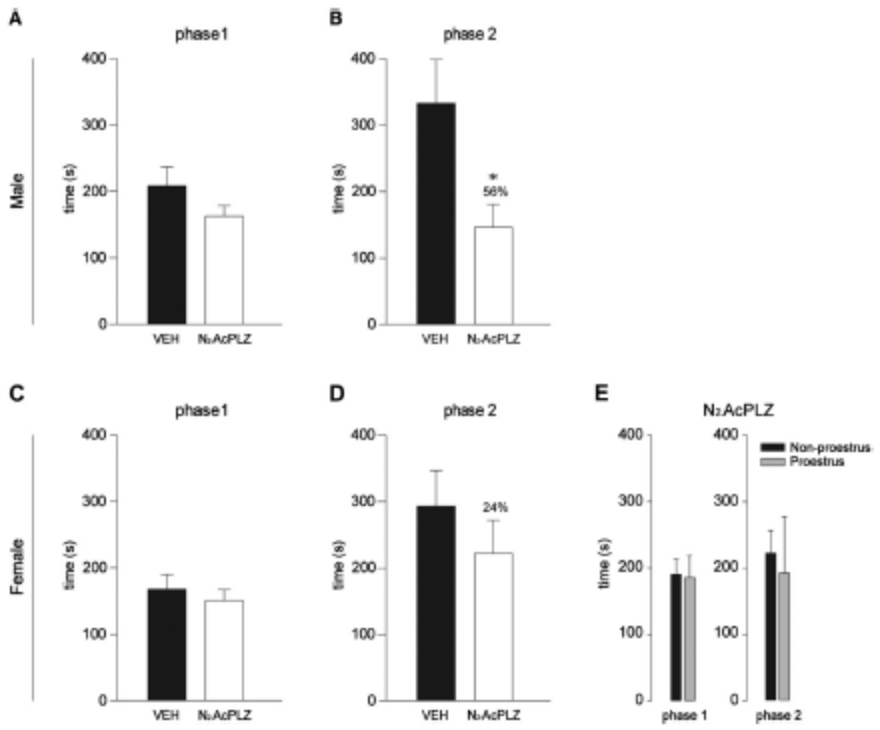

Figure 1.4. *Effect of an analogue of PLZ, N^2-AcPLZ on male and female responses to formalin.* **A, C.** Effect of N^2-AcPLZ on time spent engaging in nociceptive behaviour for male and female mice in phase 1 was observed and no differences were found. **B**. Male mice treated with N^2-AcPLZ had a significant 56% reduction in formalin induced nociceptive behavior. **D.** There was no effect of N^2-AcPLZ on time spent engaging in nociceptive behaviour for female mice phase 2, with a reduction in nociceptive behaviour of only 24%. **E.** Stage of estrus cycle did not influence formalin behaviour in female mice. *Student's t-tests, * P<0.05. All graphs are mean ±SEM.*

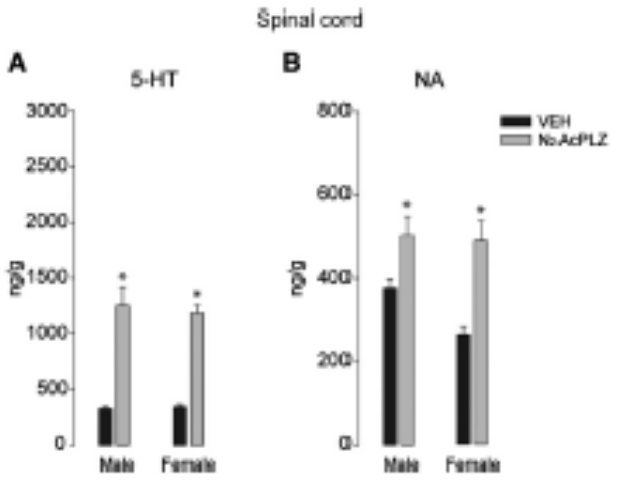

Figure 1.5. *Effect of an analogue of PLZ, N^2-AcPLZ on neurotransmitter levels in the spinal cord.*

A, B. Pretreatment with N^2-AcPLZ was found to increase 5-HT levels in the spinal cord overall in both sexes. *Two-way ANVOAs, * P<0.05. All graphs are mean ±SEM.*

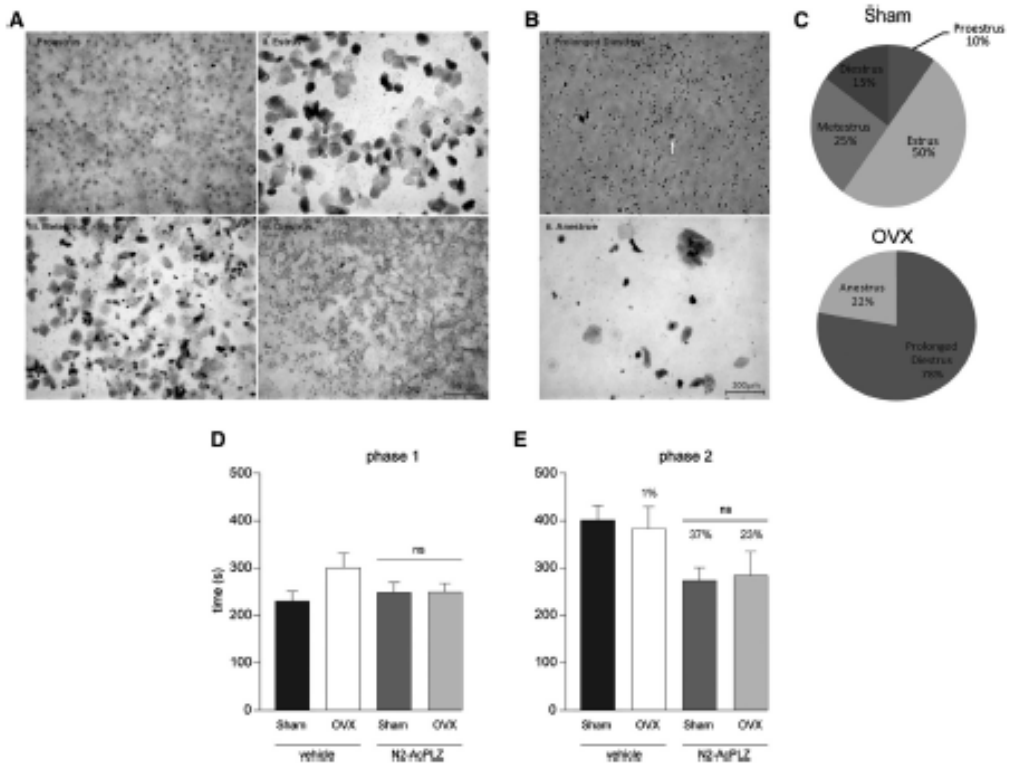

Figure 1.6. *Effect of female gonadal hormones and an analogue of PLZ, N^2-AcPLZ on formalin behaviour.*

A. Example pictures from estrus cycle stages from sham surgery female mice: proestrus (Ai), estrus (Aii), metestrus (Aiii), and diestrus (Aiv**). B.** Example pictures from ovariectomized female mice (OVX) vaginal lavage samples. Samples were categorized into 2 groups: prolonged diestrus and anestrus. Samples were classified as prolonged diestrus if there was a large number of leukocytes (white arrow) and no other discernable cell types (Bi) and anestrus (Bii) if there were no identifiable cell types. **C.** Percent of mice in estrus cycle stages. Sham surgery mice could be found in all normal stages of the estrus cycle, while ovariectomized mice did not fall into traditional categories, indicating a successful dampening of gonadal hormones. **D-E.** Time spent engaging in nociceptive behaviour during phase 1 and 2 formalin testing did not differ between groups as only 37% and 23% reduction in nociceptive behaviour was seen in N^2-AcPLZ treated Sham and OVX mice respectively. *Two-way ANVOAs, $P<0.05$. All bar graphs are mean ±SEM.*

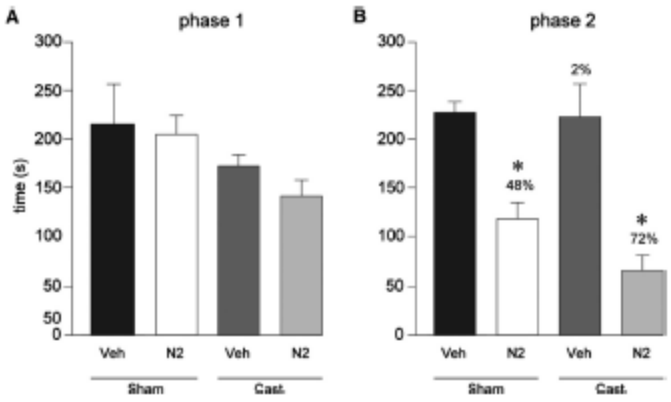

Figure 1.7. *Effect of male gonadal hormones and a compound of PLZ, N^2-AcPLZ, on formalin behaviour.*

A. All groups spent equal time engaging in nociceptive behaviour during phase 1 formalin testing. **B.** Sham surgery (Sham) and castrated mice (Cast) treated with N^2-AcPLZ (N2) demonstrated less nociceptive behaviour compared to Sham- and Cast VEH (Veh)- treated control mice controls, as seen by a 48% and 72% reduction, respectively in the surgery groups. *Two-way ANOVAs, *P<0.05. All graphs are mean ±SEM.*

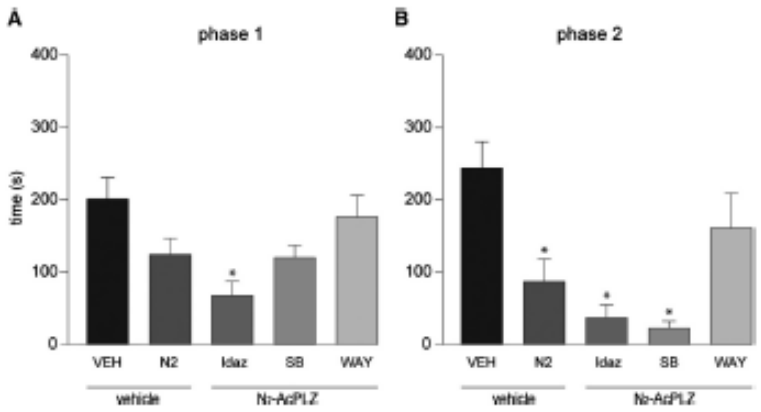

Figure 1.8. *Effect of pharmacological antagonism and a compound of PLZ, N^2-AcPLZ, on nociception in male mice.* **A.** Only treatment with N^2-AcPLZ and Idaz significantly reduced nociceptive behaviours during phase 1 formalin testing. **B.** All groups except mice treated with WAY-100635 (WAY) and N^2-AcPLZ showed reduction in nociceptive behaviour during phase 2 for formalin testing. *VEH= vehicle treated, N2= N2-AcPLZ, Idaz= Idazoxan hydrochloride, SB= SB-699551, WAY= WAY-100635. Two way ANOVAs, *P<0.05. All graphs are mean ±SEM.*

1.6 References

Aloisi AM, Ceccarelli I, Fiorenzani P, De Padova AM, Massafra C: Testosterone affects formalin-induced responses differently in male and female rats. Neurosci Lett 361: 262-264, 2004

Andurkar SV, Gendler L, Gulati A: Tramadol antinociception is potentiated by clonidine through α_2-adrenergic and I_2-imidazoline but not by edothelin ET_A receptors in mice. Eur J Pharmacol 683:109-115, 2012

Attal N, Cruccu G, Baron R, Haanpää M, Hansson P, Jensen TS, Nurmikko T, European Federation of Neurological Societies: EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. Eur J Neurol 17:1113-1188, 2010

Berkley KJ: Sex differences in pain. Behav Brain Sci 20:371-380, 1997.

Berkley KJ, Zalcman SS, Simon VR: Sex and gender differences in pain and inflammation: a rapidly maturing field. Am J Physiol Regul Integr Comp Physiol 291: R2410R244, 2006.

Besson M, Matthey A, Daali Y, Poncet A, Vuillemier P, Curatolo M, Zeilhofer HU, Desmeules J: GABAergic modulation in central sensitization in humans: a randomized placebo-controlled pharmacokinetic-pharmacodynamic study comparing clobazam with clonazepam in healthy volunteers. Pain 156: 397-404, 2015.

Bhalla S, Andurkar SV, Gulati A: Involvement of α_2-adrenoceptors, imidazoline, and endothelin-A receptors in the effect of agmatine on morphine and oxycodone-induced hypotherma in mice. Fundam Clin Pharmacol 27: 498-509, 2013.

Boerboon D, Kumar V, Boyer A, Wang Y, Lambrot R, Zhou X, Rico C, Boehm U, Paquet M, Céleste C, Kimmins S, Bernard DJ: B-catenin stabilization in gonadotropes impaires FSH synthesis in male mice in vivo. Endocrinology 156:323-333, 2015.

Bohren Y, Tessier LH, Megat S, Petitjean H, Hugel S, Daniel D, Kremer M, Fournel S, Hein L, Schlichter R, Freund-Mercier MJ, Yalcin I, Barrot M: Antidepressants suppress neuropathic pain by a peripheral β2-adrenoceptor mediated anti-TNFα mechanism. Neurobiol Dis 60:39-50, 2013.

Butkevich IP, Mikailenko VA, Vershinia EA, Otellin VA, Aloisi AM: Buspirone before prenatal stress protects against adverse effects of stress on emotional and inflammatory pain-related behaviors in infant rats: age and sex differences. Brain Res 1419:76-84, 2011.

Cervantes-Durán C, Rocha-González HI, Granados-Soto V: Peripheral and spinal 5-HT receptors participate in the pronociceptive and antinociceptive effects of fluoxetine in rats. Neurosci 252: 396-409, 2013.

Chang PC, Pollema-Mays SL, Centeno MV, Procissi D, Contini M, Baria AT, Martina M, Apkarian AV: Role of nucleus accumbens in neuropathic pain: linked multi-scale evidence in the rat transitioning to neuropathic pain. Pain 155: 1128-1139, 2014.

Chen H, Perez J, Constantopoulos E, McKee L, Regan J, Hoyer PB, Brooks HL, Konhilas J: A method to study the impact of chemically-induced ovarian failure on exercise capacity and cardiac adaptation in mice. J Vis 86: 51083, 2014.

Cicero TJ, Nock B, O'Connor L, Meyer ER: Role of steroids in sex differences in morphine-induced analgesia; activational and organizational effects. J Pharmacol Exp Ther 300: 695-701, 2002.

Cobacho N, Calle JL, Paíno CL: Dopaminergic modulation of neuropathic pain: Analgesia in rats by D2-type receptor agonist. Brain Res Bull 106: 62-71, 2014.

Coderre TJ, Melzack R: The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 12: 3665-3670, 1992.

Coderre TJ, Vaccarino AL, Melzack R: Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res 535: 155-158, 1990.

Craft RM: Sex differences in opioid analgesia: "From mouse to man". Clin J Pain 19:175-186, 2003.

Dubuisson D, Dennis SG: The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4:161-174,1977.

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC, Wallace MS: Pharmacologic management of neuropathic pain: Evidence-based recommendations. Pain 132:237-251, 2007.

Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley (III) JL: Sex, gender, and pain: A review of recent clinical and experimental findings. J Pain 10: 447-485, 2009.

Finnerup NB, Sindrup SH, Jensen TS: The evidence for pharmacological treatment of neuropathic pain. Pain 150; 573-581, 2010.

Gazerani P, Wang K, Cairns BE, Svensson P, Arendt-Nielsen L: Effects of subcutaneous administration of glutamate on pain, sensitization and vasomotor responses in healthy men and women. Pain 124: 338-348, 2006.

Gioiosa L, Chen X, Watkins R, Klanfer N, Bryant CD, Evans CJ, Arnold AP: Sex chromosome complement affects nociception in tests of acute and chronic exposure to morphine in mice. Horm Behav 53:124-130, 2008.

Gioiosa L, Chen X, Watkins R, Umeda EA, Arnold AP: Sex chromosome complement affects nociception and analgesia in newborn mice. J Pain 9: 962-969, 2008.

Greenspan JD, Craft R, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA, Mogil JS, Murphy AZ, Traub RJ, Consensus Working Group of the Sex, Gender, and Pain SIG of the IASP: Studying sex and gender differences in pain and analgesia: A consensus report. Pain 132: S26-S45, 2007.

Hache G, Guiard BP, Nguyen TH, Quesseveur G, Gardier AM, Peters D, Munro G, Coudoré F: Antinociceptive activity of the new triple reuptake inhibitor NS18283 in a mouse model of chemotherapy-induced neuropathic pain. Eur J Pain 19:322-333, 2015.

Hains BC, Everhart AW, Fullwood SD, Hulsebosch CE: Changes in serotonin, serotonin transporter expression and serotonin denervation supersensitivity: Involvement in chronic central pain after spinal hemisection in the rat. Exp Neurol 175:347-362, 2002.

Hayashi S, Taketa Y, Inoue K, Takahashi M, Matsuo S, Irie K, Watanabe G, Yoshida M: Effects of piperonyl butoxide on the female reproductive tracts in rats. J Toxicol Sci 38:891-902, 2013.

Hughes SW, Hickey L, Hulse RP, Lumb BM, Pickering AE: Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. Pain 154:1680-1690, 2013.

Juni A, Cai M, Stankova M, Waxman AR , Arout C, Klein G, Dahan A, Hruby VJ, Mogil JS, Kest B: Sex-specific mediation of opioid induced hyperalgesia by the Melanocortin-1 receptor. Anesthesiology 112:181-188, 2010.

Kang SY1, Kim CY, Roh DH, Yoon SY, Park JH, Lee HJ, Beitz AJ, Lee JH: Chemical stimulation of the ST36 accupoint reduces both formalin-induced nociceptive behaviors and spinal astrocyte activation via spinal alpha-2 adrenoceptors. Brain Res Bull. 86:412-21, 2011.

Kim SJ, Calegesan AA, Li P, Wei F, Zhuo M: Sex differences in late behavioural response to subcutaneous formalin injection in mice. Brain Res 829: 185-189, 1999

Kim JY, Tillu DV, Quinn TL, Mejia GL, Shy A, Asiedu MNK, Murad E, Schumann AP, Totsch SK, Sorge RE, Mantyh PW, Dussor G, Price TJ: Spinal dopaminergic projections control the transition to pathological pain plasticity via a D1/D5-mediated mechanism. J Neurosci 25: 6307-6317, 2015.

Kuba T, Wu HB, Nazarian A, Festa ED, Barr GA. Jenab S, Inturrisi CE, Quinones-Jenab V: Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Horm Behav 49: 441-449, 2006.

Lin C, Lin M, Cheng R, Chen S: Testosterone depletion by castration may protect mice from heat induced multiple organ damage and lethality. J Biomed Biotechnol 1-9, 2010.

Loyd DR, Murphy AZ: Sex differences in the anatomical and functional organization of the periaqueductal gray-rostral ventromedial medullary pathway in the rat: A potential circuit mediating the sexually dimorphic actions of morphine. J Comp Neurol 496: 723-738, 2006.

Loyd DR, Murphy AZ: The neuroanatomy of sexual dimorphism in opioid analgesia. Exp Neurol 259:57-63, 2014.

McCarson KE, Enna SJ: GABA Pharmacology: the search for analgesics. Neuroschem Res 39:1948-1963, 2014.

McKenna KF, Baker GB, Coutts RT: N2-Acetylphenelzine: effects on rat brain GABA, alanine and biogenic amines. Naunyn Schmiedebergs Arch Pharmacol 343: 478-482, 1991.

McLean AC, Valenzuela N, Fai S, Bennett SAL: Performing vaginal lavage, crystal violet staining, evaluation for mouse estrus cycle staging identification. J Vis Exp 67:e4389 10.3791/4389, 2012.

McManus D, Baker G, Martin I, Greenshaw A, McKenna K: Effects of the antidepressant/antipanic drug phenelzine on GABA concentrations and GABA-transaminase activity in the rat brain. Biochem Pharmacol 43: 2386-2489, 1992.

Mogil JS: Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nat Review Neurosci 13: 859-866, 2012.

Mogil JS, Sternberg WF, Kest B, Marek P, Liebeskind JC: Sex differences in the antagonism of swim-stress-induced analgesia: effects of gonadectomy and estrogen replacement. Pain 53: 17-25, 1993.

Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KV, Lariviere WR, Groce MK, Wallace MR, Kaplan L, Staud R, Ness TJ, Glover TL, Stankova M, Mayorov A, Hruby VJ, Grisel JE, Fillingim RB: The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. PNAS 100: 4867-4872, 2003.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ: Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22: 6724-6731, 2002.

Moses-Kolko EL, Price JC, Shan N,. Berqa S, Sereika SM, Fisher PM, Coleman R, Becker C, Mason NS, Loucks T, Meltzer CC: Age, sex and reproductive hormone effects on brain serotonin-1A and serotonin-2A receptor binding in a healthy population. Neuropsychopharmacology 26: 2729-2740, 2011.

Muñoz-Islas E, Vidal-Cantú GC, Bravo-Hernández M, Cervantes-Durán C, Quiñonez-Bastidas GN, Pineda-Farias JB, Barragán-Iglesias P, Granados-Soto V: Spinal 5-HT_5A receptors mediate 5-HT-induced antinociception in several pain models in rats. Pharmacol Biochem Behav. 120:25-32, 2014.

Musgrave T, Benson C, Wong G, Browne I, Tenorio G, Rauw G, Baker GB, Kerr BJ: The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE). Brain Behav Immun 25: 1677-1688, 2011.

Nag S, Mokha SS: Activation of a Gq-coupled membrane estrogen receptor rapidly attenuated α_2-adrenocetpr-induced antinociception via an ERK 1/11-dependent, non-genomic mechanism in female rats. Neurosci 267: 122-134, 2014.

Pertovaara A: Noradrenergic pain modulation. Prog Neurobiol 80:53-83, 2006.

Rahn EJ, Iannitti T, Donahue RR, Taylor BK: Sex differences in a mouse model of multiple sclerosis: neuropathic pain behaviour in females but not males and protection from neurological deficits during proestrus. Biol Sex Diff 5: 1-18, 2014.

Riley (III) JL, Robinson ME, Wise EA, Myers CD, Fillingim RB: Sex differences in the perception of noxious experimental stimuli: a meta-analysis. Pain 74:181-187, 1998.

Rocha-González HI1, Meneses A, Carlton SM, Granados-Soto V: Pronociceptive role of peripheral and spinal 5-HT7 receptors in the formalin test. Pain 117:182-92, 2005.

Roh DH, Kim HW, Yoon SY, Kang SY, Kwon YB, Cho KH, Han HJ, Ryu YH, Choi SM, Lee HJ, Beitz AJ, Lee JH: Bee venom injection significantly reduces nociceptive behavior in the mouse formalin test via capsaicin-insensitive afferents. J Pain 7:500-12, 2006.

Saarto T, Wiffen PJ: Antidepressants for neuropathic pain. Cochrane Database Syst Rev CD005454, 2007.

Sagheddu C, Aroni S, Felice M, Lecca S, Luchicchi, Melis M, Muntoni AL, Romano R, Palazzzo E, Guida F, Maione S, Pistis M: Enhanced serotonin and mesolimibic dopamine transmissions in a rat model of neuropathic pain. Neuropharmaco 97:383-393, 2015.

Sommer C: Serotonin in pain and analgesia: Actions in the periphery. Mol Neurobiol 30: 117-125, 2004.

Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS: Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. J Neurosci 31: 15450-15454, 2011.

Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, Wieskopf JS, Acland EL, Dokova A, Kadoura B, Leger P, Mapplebeck JC, McPhail M, Delany A, Wigerblad G, Schumann AP, Quinn T, Frasnelli J, Svensson CI, Sternberg WF, Mogil JS: Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 11:629-632, 2014.

Sternberg WF, Ritchie J , Mogil JS: Qualitative sex differences in κ-opioid analgesia in mice are dependent on age. Neurosci Lett 363: 178-181; 2004.

Tashira A, Bereiter DA, Thompson R, Nishida Y: GABAergic influence on temporomandibular joint-responsive spinomedullary neurons depends on estrogen status. Neurosci 259: 53-62, 2014.

Unruh AM: Gender variations in clinical pain experience. Pain 65:123-167, 1996.

Vincler M, Maixner W, Vierck CJ, Light AR: Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin. Pharmacol bichem Behav 69: 315-324, 2001.

Wichmann MW, Zellweger R, DeMaso CM, Ayala A, Chaudry IH: Mechanism of Immunosuppression in males following trauma-hemorrhage. Arch Surg 131:1186-1192, 1996.

Xu M, Kontinen VK, Kalso E: Endogenous noradrenergic tone controls symptoms of allodynia in the spinal nerve ligation model of neuropathic pain. Eur J Pharmacol 366:41-45, 1999.

Yang HW, Zhou LJ, Hu NW, Xin WJ, Liu XG: Activation of spinal D1/D5 receptors induced late phase LTP of C-fiber-evoked field potentials in rat spinal dorsal horn. J Neurophsiol 94: 961-967, 2005.

Zeilhofer HU, Möhler H, Di Lio A: GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Cell 30: 397-402, 2009.

Zhao X, Chao Y, Wang C, Zhang JF, Zhou WH, Cui WG, Ye F, Xu Y: Chronic resveratrol treatment exerts antihyperalgesic effect and corrects co-morbid depressive like behaviors in mice with mononeuropathy: Involvement of serotonergic system. Neuropharmacol 85: 131-141, 2014.

Zhao X, Xu Y, Zhao Q, Chen CR, Huang ZL: Curcumin exerts antinociceptive effects in a mouse model of neuropathic pain: descending monoamine system and opioid receptors are differentially involved. Neuropharmacol 62: 843-854, 2012.

Chapter 2: Voluntary wheel running differentially affects disease outcomes in male and female mice with experimental autoimmune encephalomyelitis

2.0 Introduction

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease of the central nervous system (CNS) characterized by demyelination and motor deficits. In addition to these motor deficits, major primary symptoms also include muscle spasticity and weakness, dysfunction in walking and balance, and a reduction in the quality of life of patients (Lublin, 2005). MS is also associated with many secondary symptoms such as cognitive dysfunction, depression, anxiety and chronic pain (Benedict et al., 2005; Brochet et al., 2009; Nourbakhsh et al., 2016). In recent years, there has been considerable interest in possible non-pharmacological interventions for MS, such as exercise, to help manage the symptoms of MS.

Although exercise was originally thought to be detrimental to patients with MS, there are now exercise recommendation guidelines for patients with MS (CSEP, 2012). Multiple studies have examined the benefits of exercise in animal models and the underlying biology behind these effects. For example, studies examining voluntary wheel running, forced treadmill running, and swimming in experimental autoimmune encephalomyelitis (EAE), the animal model used to study MS, have all shown that exercise can be effective at reducing symptom severity in the disease(Le Page et al., 1994, 1996; Rossi et al., 2009; Bernardes et al., 2013, 2016; Patel and White, 2013; Benson et al., 2015; Mifflin et al., 2015; Pryor et al., 2015; Souza et al., 2017). In addition, it has been shown that exercise can have an effect on non-motor symptoms such as pain (Benson et al., 2015).

Although it has been well established that exercise can be beneficial in EAE, very few studies have examined the effects of exercise in male mice or compared the effects in male and female mice directly. This is interesting as it has long been established that MS is a disease with a number of sex differences. For example, it is known from clinical studies that there is a much higher prevalence of MS in females (Trojano et al., 2012; Ortona et al., 2016). Sex differences in the underlying biology of the disease, such as immune cell infiltration, have also been observed. These differences are seen in both patients and in animal models of the disease and highlight the importance of considering sex as a variable in studies of potential therapeutic interventions, such as exercise, in MS (Dunn et al., 2010; Zhang et al., 2012).

Given the lack of research in the area of exercise and sex differences in MS, the present study was designed to further explore the role of voluntary exercise and sex in EAE. Thus, in the current study both male and female mice with EAE were given daily access to a running wheel for one hour per day for 30 consecutive days and the effect of voluntary exercise on disease outcomes was explored. Interestingly, the behavioural effects of daily voluntary wheel running were found to be significantly different in male and female mice. The present study therefore examined possible sex differences in the underlying biological effect of exercise by looking at markers of inflammation, neuronal damage, and oxidative stress in the spinal cord.

2.1 Materials & Methods

2.1.1 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. All C57/Bl6J mice were ordered from Charles River Canada at age 6-8 weeks. After two weeks of habituation to the housing facility, handling and baseline testing, mice underwent EAE indcution. EAE was generated in female and male mice using myelin oligodendrocytes glycoprotein (MOG) 35–55 (Peptide Synthesis Facility, University of Calgary, n=60). MOG induction was supplemented with a subcutaneous injection of 50 μg of $MOG_{35–55}$ emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1.5 mg/mL and an interaperitoneal injection of 300ng of pertussis toxin on the day of injection and two days later. Mice were kept in quarantine for 72 hours after the induction of EAE. Clinical scores were assessed daily and mice were also weighed daily. The clinical signs of EAE were graded on a four-point scale: grade 0, normal mouse; grade 1, flaccid tail (disease onset); 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. CFA control mice did not receive MOG injections, only injections of CFA and pertussis toxin (n=40).

2.1.2 Daily Voluntary Wheel Running Paradigm

Mice were given access to a cage with a running wheel for one hour a day for 30 consecutive days. Outside of the hour of daily running, all mice were conventionally housed, 5 animals per cage, with free access to food and water. For the hour of daily running, 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″/12.5 cm #61701). Distance traveled was recorded using a Schwinn ® 20 Function bike computer (model 04SW654C6PK) fitted to the running wheel. One week prior to the induction of EAE, each animal in the running group was given access to the running cage for 1 hour per day for 3 consecutive days to habituate to the running wheel and to gather baseline running data. Starting on day 4 post induction, after the quarantine period, those mice in the running EAE groups were allowed one hour of daily access to the wheel (EAE male n = 20, CFA male n=10, EAE female n=10, CFA female =10). Non-running EAE control mice were transferred in a standard cage with only bedding and a fixed running wheel for one hour a day to control for any environmental enrichment of simply having a wheel in the cage (EAE male n = 20, CFA male n=10, EAE female n=10, CFA female =10). All animals were placed within the same cage with either the running wheel/fixed wheel on consecutive days over the course of the study.

2.1.3 Histology and Immunohistochemical Analysis

Spinal cord tissue samples were collected at the chronic time point after EAE induction (day 30 post induction, n = 5 EAE run, n = 5 EAE non-running for male and female mice each). Samples were taken after mice were euthanized by an overdose of pentobarbital sodium (340 mg/ml) and then fixed via a transcardial perfusion of 0.9% saline solution followed by transcardial perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cords were then removed, post-fixed overnight and then transferred to a 30% sucrose solution in 0.1 M PB until preparation for cryostat sectioning. 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 (20 µm sections). Slides with 6-8 spinal cord sections were washed 3 times in 1x phosphate-buffered saline (PBS), blocked for 1 hour in PBS containing 0.2% Triton X-100 and 10% normal goat and/or donkey serum (depending on the antibody) and then incubated overnight at room temperature with the necessary primary antibodies. To assess T cell inflammation, reactive gliosis, and axonal damage, rat anti-CD4 (1:1000, Bio-Rad), rabbit anti-Iba-1 (1:500, Wako), and mouse anti-SMI 312/32 (1:500, Cedarlane), were used respectively. These antibodies were visualized using goat anti-rat, goat anti-mouse, or goat anti-rabbit Alexa Fluor® 488 or 594 (1:200, Invitrogen-Life Technologies) secondary antibodies

where appropriate. To visualize cellular nuclei, tissue sections were counter stained with mounting media containing fluorescence DAPI (Vector Laboratories).

Fluorescent images (5x and 10x) were visualized and captured with a Zeiss Axiocam MRm camera with a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Demyelination in tissue samples was detected using an eriochrome stain and imaged using a light microscope (Leica DMI 6000B microscope). Total cell counts for $CD4^+$ T-cell infiltration 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 of the whole lumbar spinal cord. DAPI was used in all images to ensure that each stain associated with the nucleus of a cell for counting. Confocal images of DAPI co-localization were taken at 43x magnification on a Leica DMI400B microscope to demonstrate this (Leica Microsystems, Wetzlar, Germany). Additionally, a standard total area was measured over the target region for each stain, within which cells were identified and the cell counting parameters kept constant. Images were also all taken at the same exposure to eliminate differences in background staining between sections. All measurements for cell counts are the average of 3 sections per slide, 2 slides per animal and n = 4–5 per group. For analysis of demyelination and axonal damage, areas without eriochrome stain or SMI 312/32 staining were traced on each section to calculate the total area without staining. The percent of the total area was then calculated by dividing the area of lack of staining by the total area of the slice. Images were also all taken at the same exposure for each stain. All measurements are also the average of 3 sections per slide, 2 slides per animal and n = 4–5 per group.

2.1.4 Western Blot Analysis

Western blots for Iba-1 and superoxide dismutase 2 (SOD2) were performed on whole spinal cord samples from mice. Samples were run using an SDS-page electrophoresis system from BioRAD and run on MINI-PROTEAN precast gels from BioRAD. After transfer, polyvinylidene difluoride (PVDF) membranes were blocked with 5% bovine serum albumin (BSA) in phosphate buffer solution (PBS) and then incubated with primary antibody diluted in 1% BSA overnight (Wako Rb α Iba 1 1:1,000 or Cell Signal Rb α SOD 2 1:2000). After incubation, membranes were washed twice in PBS-Tween and one time in 1xPBS. Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit, 1:10,000) and

visualized using an ECL system (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer.) Monoclonal mouse anti-βactin (1:2,000, Sigma) was used as a loading control. Blots were quantified using ImageJ software and log ratios were calculated to compare experimental groups to control non running CFA groups for each sex.

2.1.5 Superoxide Dismutase Activity Assay

Whole blood samples were collected from mice after euthanasia via a cardiac puncture. Samples were set on ice for 15-20 minutes and then centrifuged for 4 minutes at 16,000 RPM. The supernatant was then removed and stored at -80°C until needed. Activity of plasma superoxide dismutase (SOD) enzyme was measured using a colorimetric inhibition assay kit (19160 SOD determination kit, Sigma Aldrich). In the assay kit, xanthine is converted to uric acid and a superoxide anion ($2O_2^-$). The superoxide anion can react with the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) in the kit to produce a water soluble formazan dye. However, SOD can directly inhibit the activity of xanthine oxide; thus when the activity of SOD is higher, there is less superoxide anion production and less dye production. Therefore, the activity of SOD can be determined using a colorimetric method.

Samples were first diluted 10 times using the dilution buffer provided in the assay kit and then exposed to the xanthine oxidase solution (WST working solution). Percent inhibition was calculated as a measure of SOD activity using the following formula: inhibition rate %= {[(Ablank 1-Ablank3)-(Asample-Ablank2)]/(Ablank 1-Ablank 3)} x 100. In the formula, A stands for the average of three for each component and blank 1 is a control for all components of the kit minus the sample solution; blank 2 is a control for any colour in the sample, and blank 3 is a control solution with no active enzyme. Increased percent inhibition is indicative of increased SOD activity.

2.1.6 High Performance Liquid Chromatography

Spinal cords were dissected fresh (after mice were euthanized via a pentobarbital sodium injection) and flash frozen in liquid nitrogen (n=5 per experimental group). The tissue was then prepared for high performance liquid chromatography (HPLC) analysis to determine levels of glutathione (GSH) and glutathione disulfide (GSSG). The ratio of GSH/GSSG is indicative of oxidative stress, with a higher ratio indicating less stress (Schafer and Buettner, 2001). HPLC

procedures were similar to those previously used, with minor modifications as outlined below (Grant et al., 2006; Musgrave et al., 2011b).

The fresh tissue used for amino acid analysis was homogenized in 5x volume of ice-cold water. An aliquot of the diluted samples was then added to 4x ice-cold methanol, left on ice for 10 minutes and then centrifuged (12,000*g* for 4 minutes). The resulting supernatant was then diluted with distilled water to a 120-fold dilution. The resulting supernatant was then reacted with o-pthaldialdehyde and N-iso-butyryl-L-cysteine dissolved in a borate buffer. The resulting derivatives were used for analysis with a fluorescence detector set at an excitation wavelength of 344 nm and an emission wavelength of 433 m. Calibration curves for amino acids were prepared from authentic amino acid samples and generated for each individual run of samples. Of note, all water used was distilled and purified using the Milli-Q filtration system from Millipore (Billerica, Massachusetts). Methanol, tetrahydrofuran, and acetonitrile were HPLC-grade from Fisher Scientific (Pittsburgh, PA) and used in solvent solutions. All solvents were filtered using Millipore nylon membranes (0.2-µm pore size). o-Pthaldialdehyde and ascorbic acid were from Sigma-Aldrich, N-iso-butyryl-L-cysteine from NOVA Chemicals (Calgary, Alberta, Canada) and sodium borate from Fisher Scientific.

2.1.7 Statistical Analysis

Statistical analysis was carried out using two tailed t tests or one-way ANOVAs with Holm-Sidak *post hoc* tests (unless otherwise indicated) or two-way repeated measures ANOVAs where appropriate with Holm-Sidak *post hoc* tests (unless otherwise indicated). For non-parametric data, an ANOVA on ranks used or a Mann-Whitey U test, where appropriate. Significance was set at $p < 0.05$. All statistical analysis was preformed with Sigma Plot 13.0 software.

2.2 Results

2.2.1 The Effect of Voluntary Wheel Running in Male and Female Mice

Male and female mice with EAE and non-disease controls immunized only with the CFA adjuvant were given daily access for one hour to a cage with a running wheel or a cage with a fixed running wheel (non-running controls). Voluntary wheel running delayed the onset of EAE by approximately 3 days in female mice and approximately 1 day in male mice (Table 1). In addition, voluntary wheel running had a beneficial effect on the average final clinical score in male mice, with non-running male mice with EAE having a final average clinical score of 3.3±0.8 and those who ran having a score of 2.2±1.2 (Table 1). However, in female mice, running had the opposite effect. Female mice with EAE given daily access to a running wheel had an average final clinical score of 3.1±0.9 while non-running females with EAE had a final average clinical score of 2.1±1.3 (Table 1). Furthermore, the number of mice that reached a clinical score of grade 3 or 4 (indicative of severe disease) was higher in female mice who ran than those who did not (Table 1). Interestingly, the opposite pattern was seen in males, with only 25% of the male mice who ran reaching a grade of 3 or 4 after disease onset (Table 1). These results are also reflected in the clinical scores for each sex across the entire experiment (Fig. 2). We did, however, note differences in the number of mortalities in male and female EAE mice. In these experiments there were no female mortalities but there was a total of 10 mortalities in male mice with EAE (Table 1). Interestingly, this number was higher in male mice who ran, despite this group having better clinical scores on average (Table 1).

Given these initial differences, the total amount run by each group was examined to determine if differences in the amount of running between the sexes could account for the behavioural differences we observed. In both sexes, CFA control mice ran more than mice induced with MOG_{35-55} throughout the entire experiment (Fig. 1A, 1B, two way RM ANOVA, significant interaction for group x sex $F_{(1,29)}=5.563$, $P<0.001$). This is not surprising given that the ascending motor paralysis associated with disease progression prevents mice from physically running once the disease is established. Importantly however, the total distance run by both male and female CFA controls was the same (Fig. 1C, $t= 1.730$, $p=0.109$) and both male and female mice with EAE also ran the same total distance over the course of the experiment (Fig. 1D, $t= 1.431$, $p=0.164$). This suggests that it was not a difference in the overall amount of running between the sexes that accounts for the differences seen in clinical signs and disease outcomes between the sexes.

As we have found previously, daily voluntary wheel running did not improve disease progression (as measured by clinical score) in female mice ((Benson et al., 2015); Fig. 2A) In fact, a significant interaction was found for day x group with *post hoc* analyses showing that running actually increased clinical scores in female mice at certain time points after the establishment of the disease (Fig. 2A, two way RM ANOVA, significant interaction of day x group, $F_{(9,234)}$=2.296, P<0.001, *post hoc* test significant for days 14, 22, 23, 25, and 30, P<0.05 for all). However, in males, those mice who had access to a running wheel showed reduced clinical scores and had an improved disease progression overall, compared to non-running male mice with EAE (Fig. 2B, two way RM ANOVA, mina effect of group, $F_{(1,851)}$= 57.652 , P<0.001). These results demonstrate differential effects of running on clinical signs in male and female mice.

2.2.2 Changes in Inflammatory Markers with Voluntary Wheel Running: Total Spinal Cord

To gain a better understanding of the role of inflammation on the disease course in males and females with EAE, T-cell infiltration and microgliosis were examined in lumbar spinal cord sections at the end of the behavioural study. No difference was seen in CD4 positive cells between either non running and running groups in either sex (Fig. 3A&B; for males t(9)=0.103, P=0.920; for females t(7)=-0.657, P=0.532).

Iba-1 protein levels were also assessed over the entire spinal cord using Western blots. Overall, EAE increased the levels of Iba-1in the both male and female mice compared to their respective CFA controls (Fig. 3C; female two way ANOVA, main effect of disease, $F_{(1,16)}$=2, P<0.001, *post hoc* t=5.888, P<0.001; Fig. 3D; male two way ANOVA, main effect of disease $F_{(1,16)}$=9.399, P=0.007, *post hoc* t=3.066, p=0.007 Yamasaki et al., 2014). Voluntary wheel running had no effect on these levels in either sex at this chronic time point. Interestingly, when doing a direct comparison between the sexes, male mice with EAE had significantly greater levels of Iba-1 protein compared to females with the disease. (Fig. 3C&D, three way ANOVA, main effect of sex, $F_{(1,32)}$=6.384 P=0.017, *post hoc* t=2.527, P=0.017).

2.2.3 The Effect of Voluntary Wheel Running on Axonal Damage and Demyelination

We next examined markers of demyelination and axonal damage, as both are hallmarks of the pathology in EAE/MS (Fig. 4, Fig. 5). The amount of demyelination in lumbar sections of spinal

cord was examined using eriochrome C. Areas with a lack of staining are indicative of demyelination (Fig. 4A,B,D,E). Voluntary wheel running did not significantly change the total amount of demyelination in either male or female mice with EAE (Fig. 4C for females Mann Whitney U= 12.00 P=1.000; Fig. 4F for males t(8)=0.869, P=0.410). Comparing demyelination between males and females, however, revealed that males with EAE had an overall greater amount of demyelination when compared to females with the disease (Fig. 4A-F, two way ANOVA, main effect of sex, $F_{(1,16)}$=6.050, P=0.026, Tukey test q=3.478, P=0.026).

Staining with an antibody cocktail against phosphorylated and non-phosphorylated neurofilament 200 (SMI 312/32) identifies axonal loss by highlighting areas devoid of any staining (Berard et al., 2010). Voluntary wheel running did not change the amount of axonal damage seen in female mice (Fig. 5A-C). Voluntary wheel running did, however, significantly attenuate the amount of axonal loss in males when compared to EAE mice in the non-running condition (Fig. 5D-F, t(9)=2.355, P=0.0430). It should be noted again that male mice with EAE as a group (both running and non-running cohorts) had significantly more axonal loss overall compared to females (Fig. 5D-F, two way ANOVA, main effect of sex, $F_{(1,16)}$=14.276, P=0.002, Tukey test q= 5.343, P=0.002). The increased amounts of demyelination and axonal loss seen throughout the spinal cord of male mice may by indicative of the increased overall severity of clinical signs that we observed in male mice with EAE compared to females (Table 1).

2.2.4 Voluntary Wheel Running Alters Oxidative Stress Levels in Mice with EAE

Alterations in the levels of oxidative stress are known to occur in MS and to contribute to disease pathology (Gilgun-Sherki et al., 2004). In addition, it has been shown that exercise can help reduce oxidative stress in EAE (Benson et al., 2015; Souza et al., 2017). The ratio of glutathione (GSH) to glutathione disulfide (GSSG) is a useful marker of oxidative stress, with a lowered ratio being indicative of an increase in ongoing oxidative stress (Schafer and Buettner, 2001). Previous work by our group has shown that in female mice with EAE, this ratio is increased at the onset of clinical signs in mice given daily access to a running wheel, indicative of lowered levels of oxidative stress in the spinal cord (Benson et al., 2015). In the current study, we were interested in examining how this ratio is affected in the chronic stages of the disease in male and female mice with access to a running wheel. The levels of GSH increased with running in both male and female mice (Fig. 6A for females, Mann-Whitney U=0.000, P=0.008; Fig. 6B for

males t(8)=-2.860, P=0.0212). GSSG levels were however, not statistically different between groups in females (Fig. 6C) (t(7)=-0.784 P=0.479). In contrast, for males with EAE given access to a running wheel, GSSG levels were significantly lower compared to non-running controls. (Fig. 6D) (t(8)=3.595, P=0.00703). When we examined the ratio of GSH/GSSG, male mice with EAE that had daily access to the running wheel had a significantly increased GSH/GSSG ratio compared to sedentary males (Fig. 6F Mann-Whitney U=0.000 P= 0.008). This increase was not seen in female mice (Fig. 6E, Mann-Whitney U=8.000, P= 0.730). The increased ratio of GSH/GSSG in male mice with EAE given access to the running wheel is indicative of an overall reduction in oxidative stress (Fig. 6F).

Superoxide dismutase is an enzyme that catalyzes the conversion of *superoxide* (O_2^-) radicals into either oxygen (O_2) or hydrogen peroxide (H_2O_2). A reduction in this enzyme's activity is thought to be associated with increased oxidative stress (Zargari et al., 2007). We measured the activity of the SOD enzyme in plasma samples using an inhibition assay kit, where an increase in percent of inhibition of xanthine oxidase activity is indicative of increased SOD enzyme activity. We found no change in SOD activity with male mice throughout the experiment (Fig. 7B). However, we observed significant changes in SOD activity in female mice. Interestingly, reduced SOD activity was observed in mice with EAE (both running and non-running controls) as well as in non-diseased, CFA controls given access to the running wheels (Fig 6A two way ANOVA, main effect of running $F_{(1,25)}$=6.187, *post hoc* t=2.487, P=0.020 and disease $F_{(1,25)}$=14.453, *post hoc* t=3.802, P<0.001). These changes in SOD activity were also reflected in SOD protein levels as measured by western blot. Female mice with EAE have a significant decrease in SOD protein levels in the spinal cord (Fig. 7C, two way ANOVA main effect of disease $F_{(1,16)}$ =27.593, *post hoc* t= 5.253, P<0.001). In contrast, there were no significant change in SOD protein levels in male mice with EAE (Fig. 7D).

2.3 Discussion

Exercise has been shown previously to limit both the progression of clinical signs and secondary symptoms such as nociceptive hypersensitivity in EAE (Klaren et al., 2014; Benson et al., 2015; Pryor et al., 2015). However, the influence of sex on the effects of exercise in EAE has not been systematically explored. Sex is an important variable to consider as it has been implicated in many aspects of the disease, such as pain behaviour or T-cell infiltration (Dunn et al., 2010;

Zhang et al., 2012b; Rahn et al., 2014). In the current study, both male and female mice were induced with EAE and the effects of daily voluntary wheel running on disease progression was examined. We observed many striking differences between male and female mice with EAE in this paradigm. Daily voluntary wheel running had minimal (and possibly negative) effects on clinical scores in female mice with EAE over the course of the disease. Male mice with EAE given daily access to a running wheel did however, have significant improvements in clinical score. These behavioural results combined with the differences in inflammatory makers, axonal damage, demyelination, and oxidative stress between the sexes suggests that not only does running affect male and female mice with EAE differently, but that the underlying biology of the disease may differ significantly between the two sexes. These results lend support to the growing body of research showing that sex is an important variable to consider when trying to develop new therapeutics or to better understand the pathophysiology of a disease.

Although mice with EAE were able to continue running after the onset of disease, eventually there was a large difference in the amount run by non-diseased, CFA-treated controls and mice with EAE. These differences in the overall amount of running between mice treated with CFA and those induced with EAE are a result of the paralysis that develops in the EAE model which limits the capacity of mice with EAE to run in the later stages of the disease. This emphasizes the point that exercise is not a curative treatment, but may instead be a non-pharmacological way to modify the severity of clinical signs of the disease. We did, however, find that running delayed the onset of disease in female mice by approximately three days, a result seen previously by our group and one that has been replicated by others (Table 1; (Le Page et al., 1994, 1996; Benson et al., 2015; Bernardes et al., 2016). Curiously, this delay was not found in male mice. These results highlight an important variable to consider when looking at exercise as a potential therapeutic: timing of the intervention. In the current study, and in many others, the exercise intervention is given after the induction of EAE, however whether or not this is the best timing for an exercise intervention is not yet known. One study has explored this question by looking at delivering an exercise intervention only during periods of remission and found that there was no effect of exercise of clinical outcomes (Klaren et al., 2016). However, studies looking at exercise interventions delivered prior to or directly after disease induction seem to be effective (Rossi et al., 2009; Bernardes et al., 2013, 2016; Benson et al., 2015; Pryor

et al., 2015). This suggests that there may be a critical time period for exercise to have beneficial effects in EAE.

We also found many differences between our male and female groups that reflected sex differences often seen in the clinic. For example, we had a mortality rate of 25% in male mice with EAE but a 0% mortality rate in females with the disease. This increase in mortality may be reflective of the clinical studies demonstrating that disease progression is often more severe in males with MS (Bove and Chitnis, 2013). Importantly, one of the only other studies looking at exercise in male mice with EAE also reported higher rates of mortality (Pryor et al., 2015). Furthermore, the average clinical score was significantly lower in females with EAE compared to male mice and fewer female mice reached a severe disease score of clinical grade 3 or 4. Again these results are reflective of the sex differences seen in clinical populations where, although more females are affected with MS, the disease is often more severe in males (Bove and Chitnis, 2013).

Although male mice with EAE had a more severe disease overall, male mice given daily access to a running wheel had significantly lower clinical scores compared to non-running controls with the disease. This is in agreement with previous research showing that exercise can improve clinical scores in male mice (Pryor et al., 2015). Interestingly, we observed the opposite pattern for females with EAE. Female mice with EAE who had access to the running wheels had significantly higher clinical scores in the chronic stages of the experiment when compared to non-running controls. This is in contrast to other reports in the field (Le Page et al., 1994, 1996; Rossi et al., 2009; Bernardes et al., 2013, 2016). These differences in behavioural outcomes are most likely due to differences in the exercise paradigms used in the different studies. Several reports have directly compared different exercise paradigms and have shown different behavioural outcomes depending on the paradigm used. For example, in a study by Le Page and colleagues that compared severe/intense exercise with constant speed exercise, they demonstrated that only the intense exercise paradigm was effective at reducing clinical scores in mice with EAE (Le Page et al., 1996).

When we evaluated T-cell infiltration and microglia activation we detected significant sex differences between the running groups. There was no difference in the total amount of T cells over the entire spinal cord section in either sex, but significantly more microgliosis in male mice. This may suggest it is the increased microgliosis in male mice that is contributing to their

more severe disease profile. This increase in inflammation in male mice with EAE may also be contributing to the increased damage seen in the spinal cord in these mice. For example, we also found interesting sex differences with regard to neuronal injury between male and female mice with EAE. We found that there was significantly more axonal loss over the entire spinal cord in male mice compared to females. Again, this is a factor that likely contributes to the higher clinical scores we observed in male mice compared to females. Running did, however, significantly reduce the amount of axonal damage in the spinal cord of male mice. Given the overall increase in the amount of axonal damage seen in male mice, this reduction with running may underlie the improvements in clinical score that we observed in male mice. Furthermore, we also found significant reductions in the amount of demyelination in males with EAE that were given access to the running wheels. This is another indication that daily exercise can lead to an overall improvement in the health of spinal cord axons in males with EAE.

There have been many studies linking central nervous system damage to oxidative stress in MS (Gilgun-Sherki et al., 2004; Bishop et al., 2009; Davitashvili et al., 2012). Exercise has been shown to improve oxidative stress in multiple studies (Benson et al., 2015; Souza et al., 2017). Thus, we next examined markers of oxidative stress to determine if any of the behavioural improvement that we observed in males with EAE was related to improvements in the levels of oxidative stress. GSH is a critical antioxidant that is thought to be protective due to its oxygen and radical scavenging capabilities (Pompella et al., 2003). When oxidized, GSH becomes GSSG. Increased oxidative stress leads to an increase in the amount of GSSG and is associated with a decrease in the GSH/GSSG ratio (Schafer and Buettner, 2001). Exercise in the EAE model has been previously shown to increase GSH levels and is associated with improvements in clinical outcomes (Benson et al., 2015; Souza et al., 2017). Our findings corroborate these results, with increases in the GSH/GSSG ratio in male mice who ran and increases in GSH levels in all mice given access to the running wheels. Given that an increase in the GSH/GSSG ratio is associated with improved oxidative stress, it is likely that the reduced axonal injury and demyelination in male mice given access to the running wheels is associated with improved oxidative stress management.

Superoxide dismutase (SOD) is an enzyme that catalyzes the conversion of superoxide to hydrogen peroxide. There are three forms of the SOD enzyme, Cu/Zn- SOD which is cytosolic (SOD 1), mitochondrial SOD (SOD 2), and SOD3 which is only found in extracellular

compartments. SOD activity has been found to decrease with EAE (Miller et al., 2013; Polachini et al., 2016). We found that the activity of the SOD enzyme and its actual protein level were also significantly affected in female mice with the disease but that SOD levels and the enzymatic activity remain stable in males. The significant reduction in SOD enzyme activity and protein levels in female mice suggest that females are unable to recruit this important anti-oxidant pathway in EAE. The lack of change in male mice might suggest that this pathway is not disrupted in male mice with EAE and thus does not contribute to disease pathology in male mice.

In female mice, it is possible that exercise may not be as beneficial in the chronic stages of EAE compared to its effects in males. This is especially true in the present study, as we found an increase in the clinical scores at the chronic time point of the experiment in female mice who ran. Although a recent study has shown that exercise can improve markers of oxidative stress at a chronic time point (Souza et al., 2017), it is important to consider differences in the exercise paradigms used between the different studies. Both strength and endurance training were used by Souza and colleagues, but mice only underwent training for a total of four weeks and most mice would not have been exercising once the disease was established (Souza et al., 2017). In our paradigm, mice exercised well past disease onset, an approach that is not often seen in the field. These differences are important to consider as our data suggest that exercise in the chronic stage of the disease may become stressful, particularly in female mice, thereby increasing oxidative stress instead of reducing it. This may have contributed to the increased clinical score seen in the present study in female mice who ran. Furthermore, several other studies have shown that strenuous exercise can increase the generation of reactive oxygen species (Richters et al., 2011; Crane et al., 2013). A study looking at SOD 2 -/- mice found that exercise exacerbated mitochondrial protein dysfunction in muscle, and suggested that one should be cautious when suggesting exercise in a population with mitochondrial dysfunction (Crane et al., 2013). This is important to consider as MS is a disease with known deficits in mitochondrial function.

2.4 Conclusions

The present study showed that daily voluntary wheel running has different effects on the symptoms of EAE in male and female mice. These differences are likely due to different inflammatory profiles that trigger different pathological changes in the CNS between male and female mice. Furthermore, it is likely that these changes are critically linked to differences in

oxidative stress and how it is managed in males and females. The differences we describe highlight the need to study both male and female subjects in MS research in order to better understand and treat this disease.

2.5 Figures

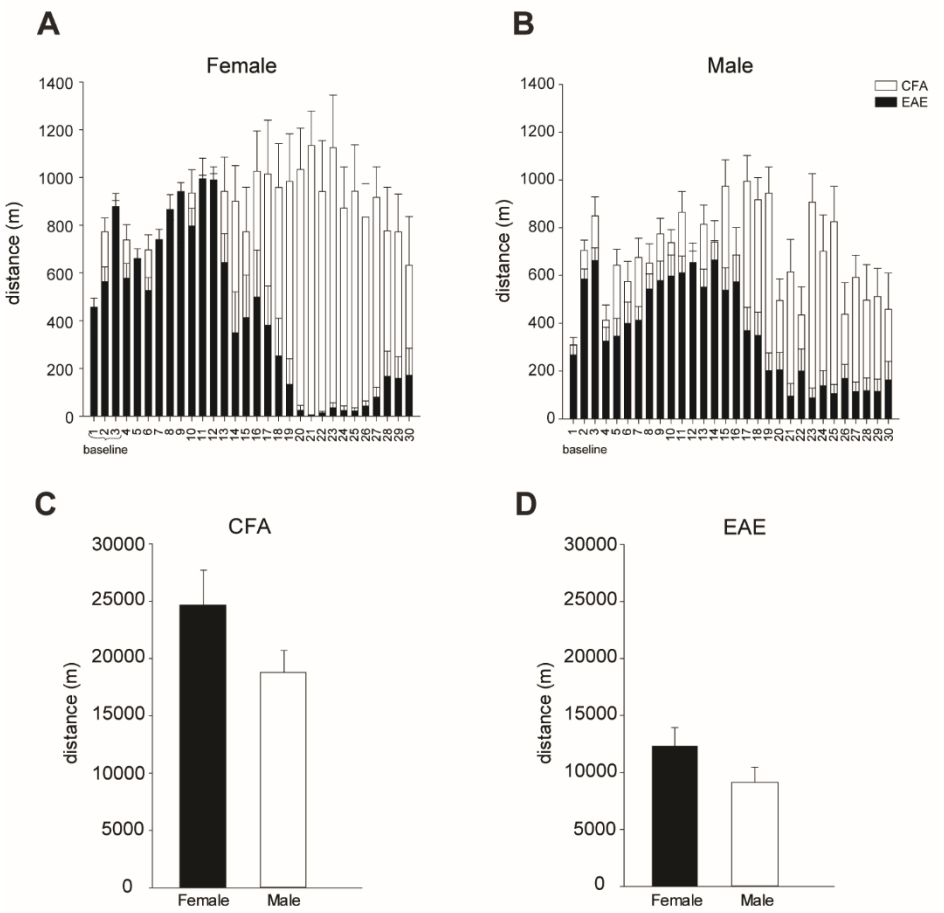

Figure 2.1. *Average distance run per day.* (A&B) Overall, CFA mice ran more compared to EAE mice in both sexes. (C) No difference was found in total distance run between male and female CFA controls or between male and female EAE mice. (*EAE= experimental autoimmune encephalomyelitis, CFA= complete Freud's adjuvant. All graphs are mean ± SEM)*

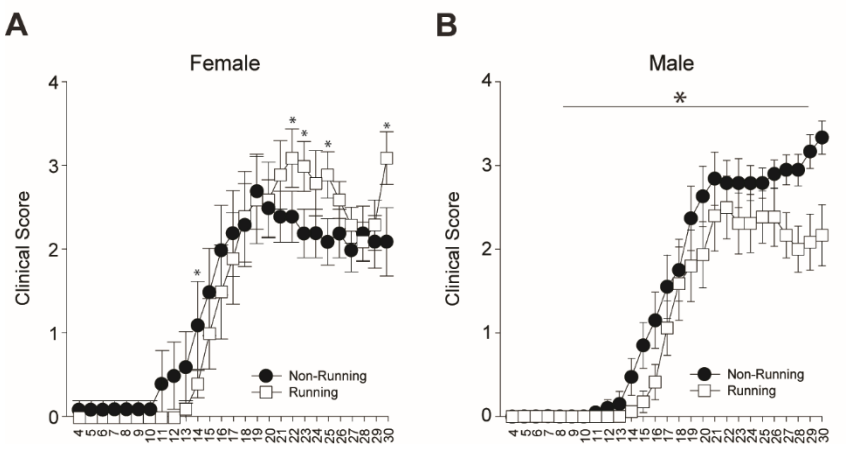

Figure 2.2. *Effect of voluntary wheel running on clinical score*. A clinical score of 0 indicates no signs of disease, 1 indicates a flaccid tail and disease onset, 2 hindlimb weakness, 3, hindlimb paralysis, and 4 complete forelimb and hindlimb paralysis. (A) Average clinical score in female mice with EAE each day. A significant interaction of day x group was found, with females who ran having significantly different scores from those who did not run on days 14, 22, 23, 25, and 30*. (B) Average clinical score per day of testing in male mice with EAE. Males mice who ran had improved clinical scores overall when compared to non running EAE mice. *(*P<0.05, Two Way RM ANOVA, EAE= experimental autoimmune encephalomyelitis. All graphs are mean ± SEM)*.

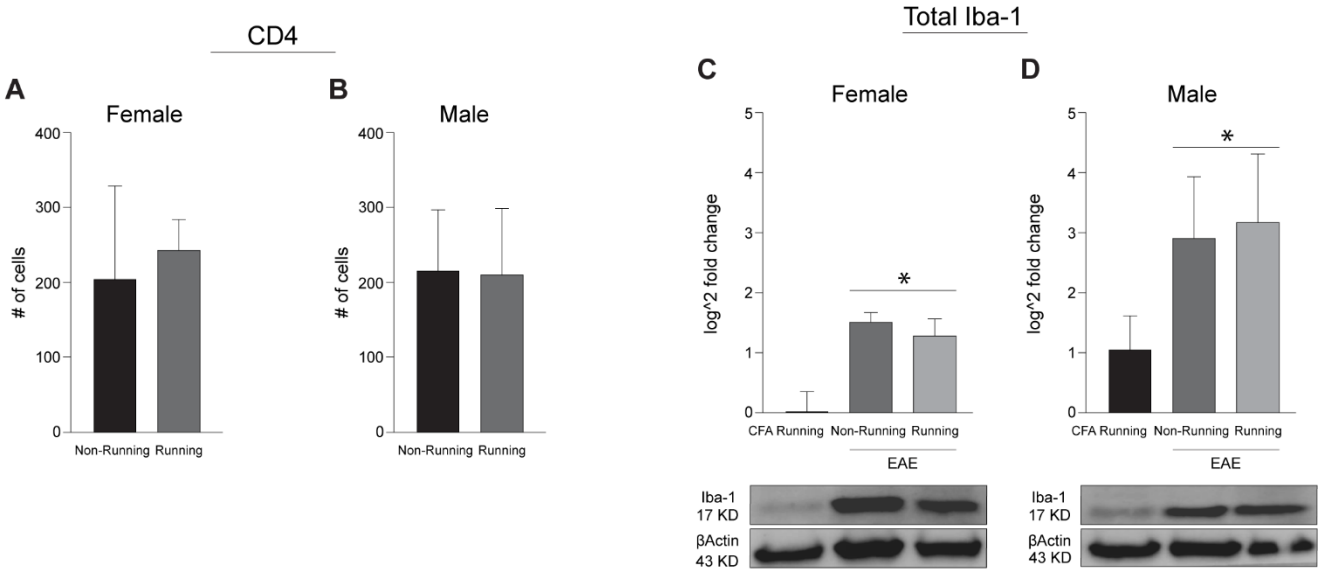

Figure 2.3. *Effect of voluntary wheel running on immune markers.* (A) Number of T-cells in the lumbar spinal cord of EAE female mice who ran and did not run. There were no differences found in the total number of CD4+ cells found. (B) Number of T-cells in the lumbar spinal cord of EAE male mice who ran and did not run. There were no differences found in the total number of CD4+ cells found. (C) Total Iba-1 proteins levels measured in whole spinal cord via western blots in female micce. Log change was calculated by comparing experimental groups to female non-running CFA controls. EAE increased the amount of protein for IBA-1 in female EAE mice, indicating increased microgliosis with disease. Running did not affect the amount of Iba-1 protein. (D)Total Iba-1 proteins levels measured in male mice. Log change was calculated by comparing experimental groups to male non-running CFA controls. EAE increased the amount of protein for IBA-1 in male EAE mice, indicating increased microgliosis with disease. Running did not affect the amount of Iba-1 protein in male mice. (C&D) Sample western blots for Iba-1 and control actin are shown for both sexes. Male mice with EAE also had more Iba-1 protein compared to female with EAE in the spinal cord. *(*P<0.05, t tests, EAE non-running n=5; EAE run n=5 in males & females. All graphs are mean ± SEM).*

Figure 4

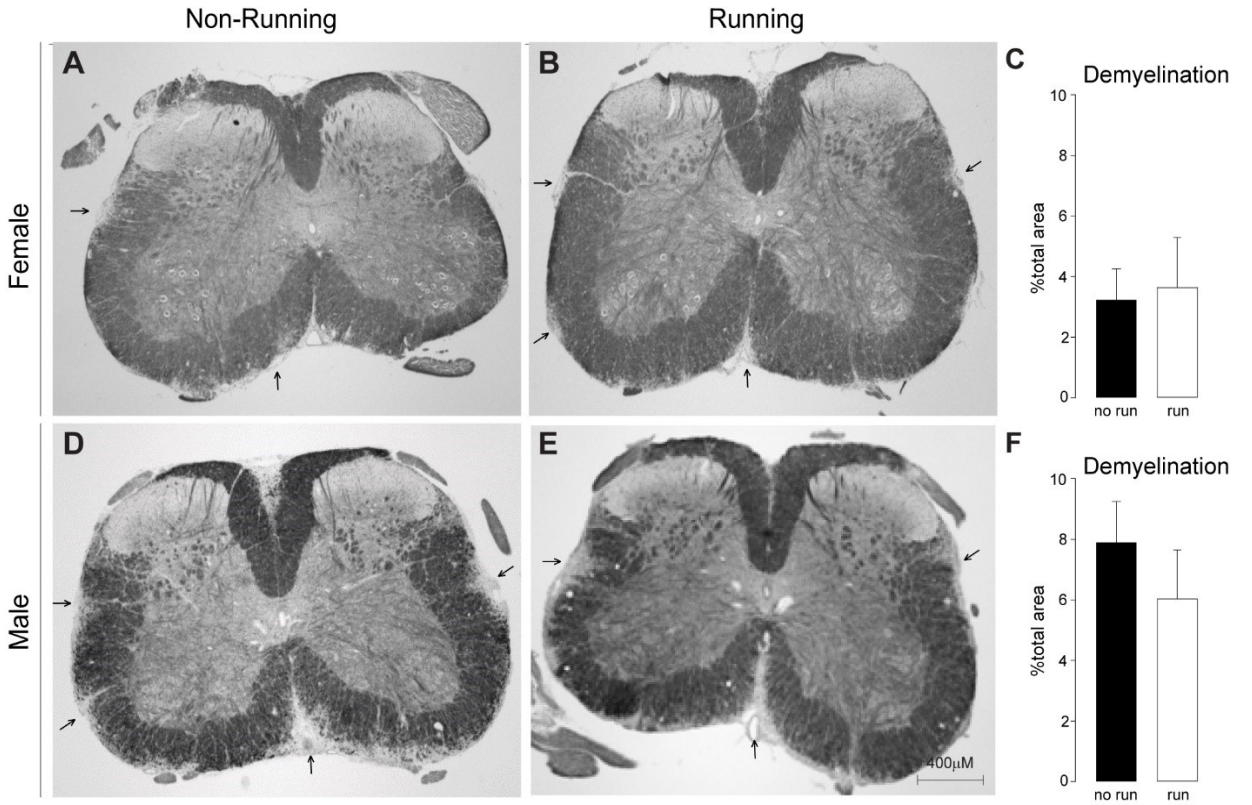

Figure 2.4. *Effect of voluntary wheel running on demyelination.* (A&B) Representative 5x female lumbar spinal cord images stained with Eriochrome stain to measure demyelination. Arrows indicate areas of demyelination. (C) Voluntary wheel running did not change the amount of demyelination in females with EAE. (D&E) Representative 5x male lumbar spinal cord images stained with Eriochrome stain to measure demyelination. Arrows indicate areas of demyelination. (F) Voluntary wheel running did not change the amount of demyelination in males with EAE either. However, males had more demyelination overall when compared to female mice. *(*P<0.05, t tests, EAE non-running n=5; EAE run n=5 in males & females. All graphs are mean ± SEM).*

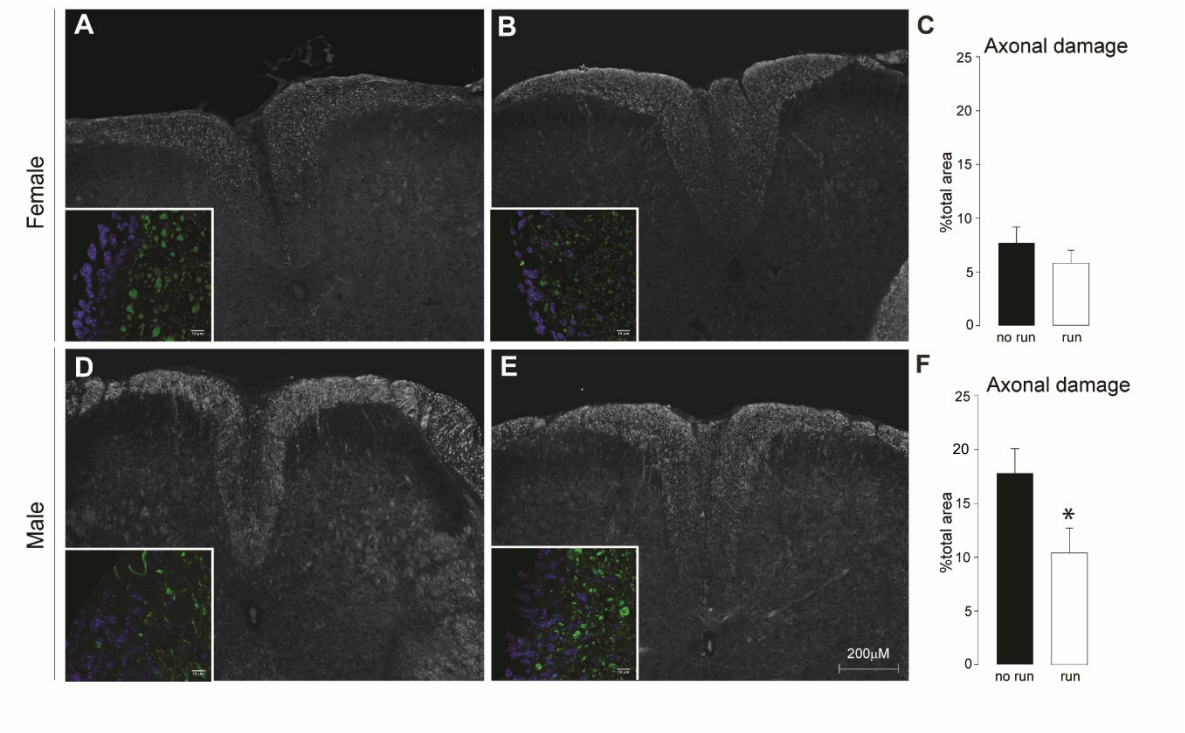

Figure 2.5. *Effect of voluntary wheel running on axonal damage.* (A&B) Representative 10x images from female lumbar section of staining with phosphorylated and non-phosphorylated neurofilament 200 (SMI 312-SMI32). Confocal inset showed lack of co-localization of axonal staining with both SMI312-SIM32 (green) with DAPI (blue), a marker for cellular nuclei, as expected for axonal labelling. Dashes outline areas of axonal damage. (C) Voluntary wheel running did not significantly alter axonal damage in female EAE mice. (D&E) Representative 10x images from male lumbar section of staining with phosphorylated and non-phosphorylated neurofilament 200 (SMI 312-SMI32). Confocal inset showed lack of co-localization of axonal staining with both SMI312-SIM32 (green) with DAPI (blue), a marker for cellular nuclei, as expected for axonal labelling. Dashes outline areas of axonal damage. F(F) Voluntary wheel running significantly decreased the amount of axonal damage seen in male mice. *(*P<0.05, t tests, EAE non-running n=5; EAE run n=5 in males & females. All graphs are mean ± SEM).*

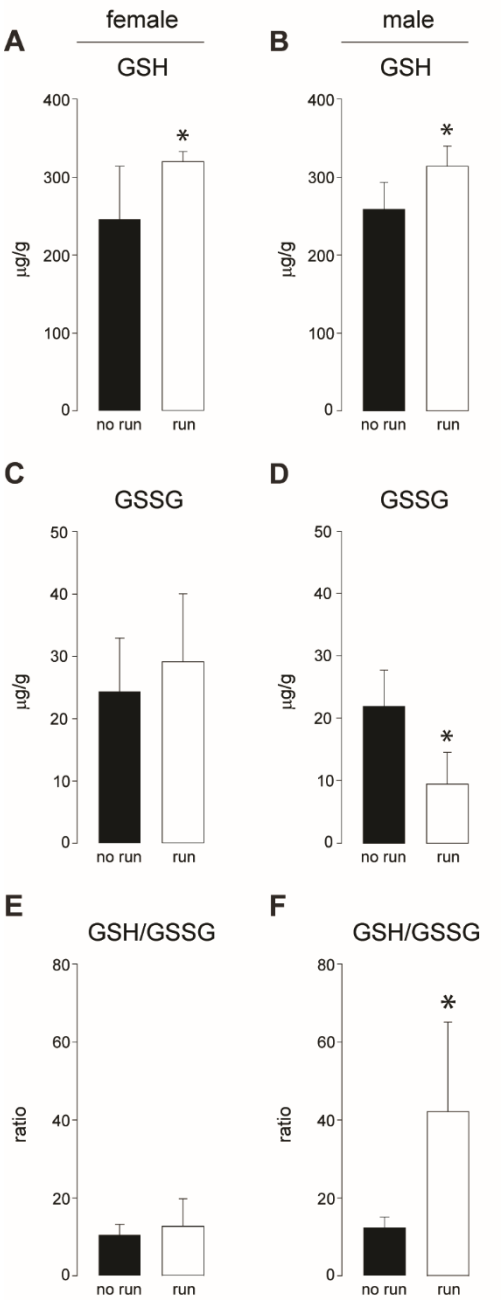

Figure 2.6. *Oxidative stress measures in running and non running mice.* (A&B) GSH levels as measured by HPLC in the spinal cord. Running increased levels of GSH in female and male mice. (C) GSSG levels in the spinal cord as measured by HPLC. No significant differences were found between groups for female mice. (D) GSSG was found to be significantly reduced in male mice with EAE who ran compared to non-runners. (E) Ratio of GSH to GSSG in the whole spinal cord as measured by HPLC. An increased ratio is indicative of reduced oxidative stress. No change was seen in female mice with running. (F) Males who ran had an increased GSH/ GSSG ratio compared to those who did not run. *(*P<0.05, t tests, EAE non-running n=5; EAE run n=5 in males & females, GSH= glutathione, GSSG=glutathione disulfide, HPLC= high performance liquid chromatography. All graphs are mean ± SEM).*

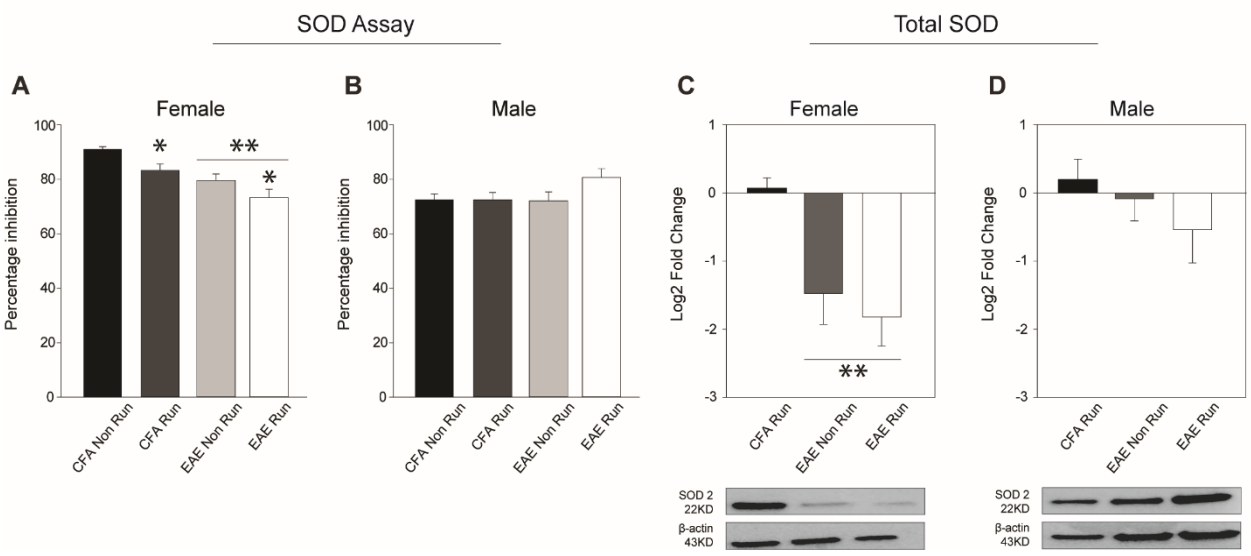

Figure 2.7. *Effect of voluntary wheel running on superoxide dismutase activity and protein level.* (A&B) SOD activity levels were measured using an inhibition assay kit and percent inhibition of xanthine oxidase activity was calculated. (A)Female mice with EAE had reduced SOD activity levels compared to CFA control mice. Female mice who ran also had reduced activity levels compared to groups who did not run. (B) No change in SOD activity was seen in male mice in any group. (C&D) Western blots were used to measure SOD2 protein levels in the spinal cord of male and female mice. Log change was calculated by comparing experimental groups to sex specific non-running CFA controls. Representative blots for SOD2 and control actin are shown for each sex. (C) Female mice with EAE had reduced protein levels of SOD2 compared to CFA controls. (D) No changes in SOD2 protein levels were seen in male mice. *(*, **, #P<0.05, Two Way ANOVAs, CFA running n= 5, EAE non-running n=5; EAE run n=5 in males & females , SOD 2= superoxide dismutase 2. All graphs are mean ± SEM).*

2.6 Table 2: *Differences between disease parameters in female and male mice.*

Disease Parameter	Females	Males
Days to Onset	Non-Runners: 14.1±4.3 Runners: 16.6±3.0 Overall: 2.6±1.2	Non-Runners:17.3±3.5 Runners: 18.3±2.8 Overall: 3.0±1.1
Average Clinical Score	Non-Runners: 2.1±1.3 Runners: 3.1±1.0 Total: 11 (55%)	Non-Runners: 3.3±0.8 Runners: 2.2±1.3 Total: 19 (63%)
Number of mice at clinical score of 3 or 4 at end of experiment	Non-Runners: 3 (30%) Runners: 8 (80%) Overall: 0	Non-Runners: 16 (88%) Runners: 3 (25%) Overall: 10 (25%)
Number of Mortalities	Non-Runners: 0 Runners: 0	Non-Runners: 2(10%) Runners: 8(40%)

*Values are mean ± SD

2.7 References

Benson, C., Paylor, J. W., Tenorio, G., Winship, I., Baker, G., & Kerr, B. J. (2015). Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE). *Experimental Neurology, 271*, 279-290. doi:10.1016/j.expneurol.2015.05.017 [doi]

Berard, J. L., Wolak, K., Fournier, S., & David, S. (2010). Characterization of relapsing-remitting and chronic forms of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Glia, 58*(4), 434-445. doi:10.1002/glia.20935 [doi]

Bernardes, D., Brambilla, R., Bracchi-Ricard, V., Karmally, S., Dellarole, A., Carvalho-Tavares, J., & Bethea, J. R. (2016). Prior regular exercise improves clinical outcome and reduces demyelination and axonal injury in experimental autoimmune encephalomyelitis. *Journal of Neurochemistry, 136 Suppl 1*, 63-73. doi:10.1111/jnc.13354 [doi]

Bernardes, D., Oliveira-Lima, O. C., Silva, T. V., Faraco, C. C., Leite, H. R., Juliano, M. A., . . . Carvalho-Tavares, J. (2013). Differential brain and spinal cord cytokine and BDNF levels in experimental autoimmune encephalomyelitis are modulated by prior and regular exercise. *Journal of Neuroimmunology, 264*(1-2), 24-34. doi:10.1016/j.jneuroim.2013.08.014 [doi]

Bishop, A., Hobbs, K. G., Eguchi, A., Jeffrey, S., Smallwood, L., Pennie, C., . . . Estevez, A. G. (2009). Differential sensitivity of oligodendrocytes and motor neurons to reactive nitrogen species: Implications for multiple sclerosis. *Journal of Neurochemistry, 109*(1), 93-104. doi:10.1111/j.1471-4159.2009.05891.x [doi]

Bove, R., & Chitnis, T. (2013). Sexual disparities in the incidence and course of MS. *Clinical Immunology (Orlando, Fla.), 149*(2), 201-210. doi:10.1016/j.clim.2013.03.005 [doi]

Brochet, B., Deloire, M. S., Ouallet, J. C., Salort, E., Bonnet, M., Jove, J., & Petry, K. G. (2009). Pain and quality of life in the early stages after multiple sclerosis diagnosis: A 2-year

longitudinal study. *The Clinical Journal of Pain, 25*(3), 211-217. doi:10.1097/AJP.0b013e3181891347 [doi]

Canadian Society for Exercise Physiology. (2012). Canadian physical activity guidelines for adults with multiple sclerosis. Retrieved from http://www.csep.ca/CMFiles/Guidelines/specialpops/CSEP_MS_PAGuidelines_adults_en.pdf

Crane, J. D., Abadi, A., Hettinga, B. P., Ogborn, D. I., MacNeil, L. G., Steinberg, G. R., & Davitashvili, D., Beridze, M., Shakarishvili, R., Kiziria, M., & Sanikidze, T. (2012). The role of endogenous antiradical protective system in multiple sclerosis. *Georgian Medical News, 205*, 11-19.

Dunn, S. E., Bhat, R., Straus, D. S., Sobel, R. A., Axtell, R., Johnson, A., . . . Steinman, L. (2010). Peroxisome proliferator-activated receptor delta limits the expansion of pathogenic Th cells during central nervous system autoimmunity. *The Journal of Experimental Medicine, 207*(8), 1599-1608. doi:10.1084/jem.20091663 [doi]

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2004). The role of oxidative stress in the pathogenesis of multiple sclerosis: The need for effective antioxidant therapy. *Journal of Neurology, 251*(3), 261-268. doi:10.1007/s00415-004-0348-9 [doi]

Grant, S. L., Shulman, Y., Tibbo, P., Hampson, D. R., & Baker, G. B. (2006). Determination of D-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences, 844*(2), 278-282. doi:S1570-0232(06)00587-3 [pii]

Klaren, R. E., Motl, R. W., Woods, J. A., & Miller, S. D. (2014). Effects of exercise in experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis). *Journal of Neuroimmunology, 274*(1-2), 14-19. doi:10.1016/j.jneuroim.2014.06.014 [doi]

Klaren, R. E., Stasula, U., Steelman, A. J., Hernandez, J., Pence, B. D., Woods, J. A., & Motl, R. W. (2016). Effects of exercise in a relapsing-remitting model of experimental autoimmune encephalomyelitis. *Journal of Neuroscience Research,* doi:10.1002/jnr.23783 [doi]

Le Page, C., Bourdoulous, S., Beraud, E., Couraud, P. O., Rieu, M., & Ferry, A. (1996). Effect of physical exercise on adoptive experimental auto-immune encephalomyelitis in rats. *European Journal of Applied Physiology and Occupational Physiology, 73*(1-2), 130-135.

Le Page, C., Ferry, A., & Rieu, M. (1994). Effect of muscular exercise on chronic relapsing experimental autoimmune encephalomyelitis. *Journal of Applied Physiology (Bethesda, Md.: 1985), 77*(5), 2341-2347.

Lublin, F. (2005). History of modern multiple sclerosis therapy. *Journal of Neurology, 252 Suppl 3*, iii3-iii9. doi:10.1007/s00415-005-2010-6 [doi]

Miller, E., Walczak, A., Majsterek, I., & Kedziora, J. (2013). Melatonin reduces oxidative stress in the erythrocytes of multiple sclerosis patients with secondary progressive clinical course. *Journal of Neuroimmunology, 257*(1-2), 97-101. doi:10.1016/j.jneuroim.2013.02.012 [doi]

Nourbakhsh, B., Julian, L., & Waubant, E. (2016). Fatigue and depression predict quality of life in patients with early multiple sclerosis: A longitudinal study. *European Journal of Neurology,* doi:10.1111/ene.13102 [doi]

Ortona, E., Pierdominici, M., Maselli, A., Veroni, C., Aloisi, F., & Shoenfeld, Y. (2016). Sex-based differences in autoimmune diseases. *Annali Dell'Istituto Superiore Di Sanita, 52*(2), 205-212. doi:10.4415/ANN_16_02_12 [doi]

Patel, D. I., & White, L. J. (2013). Effect of 10-day forced treadmill training on neurotrophic factors in experimental autoimmune encephalomyelitis. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme, 38*(2), 194-199. doi:10.1139/apnm-2012-0303 [doi]]

Polachini, C. R., Spanevello, R. M., Zanini, D., Baldissarelli, J., Pereira, L. B., Schetinger, M. R., . . . Morsch, V. M. (2016). Evaluation of delta-aminolevulinic dehydratase activity, oxidative stress biomarkers, and vitamin D levels in patients with multiple sclerosis. *Neurotoxicity Research, 29*(2), 230-242. doi:10.1007/s12640-015-9584-2 [doi]

Pompella, A., Visvikis, A., Paolicchi, A., De Tata, V., & Casini, A. F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology, 66*(8), 1499-1503. doi:S0006295203005045 [pii]

Pryor, W. M., Freeman, K. G., Larson, R. D., Edwards, G. L., & White, L. J. (2015). Chronic exercise confers neuroprotection in experimental autoimmune encephalomyelitis. *Journal of Neuroscience Research, 93*(5), 697-706. doi:10.1002/jnr.23528 [doi]

Rahn, E.J., Iannitti, T., Donahue, R.R., & Taylor, B.K. (2014). Sex differences in a mouse model of multiple sclerosis neuropathic pain behavior in females but not males and protection from neurological deficits during proestrus. Biology of Sex Differences, 5(1),4, doi: 10.1186/2042-6410-5-4 [doi]

Richters, L., Lange, N., Renner, R., Treiber, N., Ghanem, A., Tiemann, K., . . . Brixius, K. (2011). Exercise-induced adaptations of cardiac redox homeostasis and remodeling in heterozygous SOD2-knockout mice. *Journal of Applied Physiology (Bethesda, Md.: 1985), 111*(5), 1431-1440. doi:10.1152/japplphysiol.01392.2010 [doi]

Rossi, S., Furlan, R., De Chiara, V., Musella, A., Lo Giudice, T., Mataluni, G., . . . Centonze, D. (2009). Exercise attenuates the clinical, synaptic and dendritic abnormalities of experimental autoimmune encephalomyelitis. *Neurobiology of Disease, 36*(1), 51-59. doi:10.1016/j.nbd.2009.06.013 [doi]

Schafer, F. Q., & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology & Medicine, 30*(11), 1191-1212. doi:S0891584901004804 [pii]

Souza, P. S., Goncalves, E. D., Pedroso, G. S., Farias, H. R., Junqueira, S. C., Marcon, R., . . . Dutra, R. C. (2017). Physical exercise attenuates experimental autoimmune encephalomyelitis by inhibiting peripheral immune response and blood-brain barrier disruption. *Molecular Neurobiology,* 54(6), 4723-4737. doi:10.1007/s12035-016-0014-0 [doi]

Trojano, M., Lucchese, G., Graziano, G., Taylor, B. V., Simpson, S.,Jr, Lepore, V., . . . MSBase Study Group and the New Zealand MS Prevalence Study Group. (2012). Geographical variations in sex ratio trends over time in multiple sclerosis. *PloS One, 7*(10), e48078. doi:10.1371/journal.pone.0048078 [doi]

Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A. M., Cialic, R., . . . Ransohoff, R. M. (2014). Differential roles of microglia and monocytes in the inflamed central nervous system. *The Journal of Experimental Medicine, 211*(8), 1533-1549. doi:10.1084/jem.20132477 [doi]

Zargari, M., Allameh, A., Sanati, M. H., Tiraihi, T., Lavasani, S., & Emadyan, O. (2007). Relationship between the clinical scoring and demyelination in central nervous system with total antioxidant capacity of plasma during experimental autoimmune encephalomyelitis development in mice. *Neuroscience Letters, 412*(1), 24-28. doi:S0304-3940(06)00836-6 [pii]

Zhang, M. A., Rego, D., Moshkova, M., Kebir, H., Chruscinski, A., Nguyen, H., . . . Dunn, S. E. (2012). Peroxisome proliferator-activated receptor (PPAR)alpha and -gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way. *Proceedings of the National Academy of Sciences of the United States of America, 109*(24), 9505-9510. doi:10.1073/pnas.1118458109 [doi]

Chapter 3: Effect of Voluntary Wheel Running on Neuroactive Steroid Levels in Murine Experimental Autoimmune Encephalomyelitis

3.0 Introduction

Multiple sclerosis (MS) is a devastating demyelinating autoimmune disease primarily associated with motor dysfunction. Its etiology and biological mechanisms are not yet fully understood. Neuroactive steroids (NASs) have recently been implicated in several aspects of MS symptomology and progression (Purdy et al., 1991; Savettieri et al., 2004; Caruso et al., 2010, 2014; Giatti et al., 2010; Orefice et al., 2016). NASs are rapidly acting steroids that produce nongenomic effects by acting as allosteric modulators at neurotransmitter receptors, especially the NMDA subtype of glutamate receptors and/or gamma-aminobutyric acid-A (GABA-A) receptors (Tuem and Atey, 2017). NASs can come from the periphery or they may be synthesized from cholesterol in the brain; in the latter case they are termed *neurosteroids*. The two terms will be used interchangeably in this paper. Various NASs have been reported to have neuroprotective actions and may have effects on cell growth, differentiation and myelination (Tuem and Atey, 2017). Some NASs have also been reported to have anti-inflammatory actions and have been proposed to play a role in a number of neurologic, psychiatric and gastrointestinal disorders (see (Tuem and Atey, 2017) for a review).

Clinical reports have demonstrated that there are significant differences in the levels of NASs in the cerebral spinal fluid (CSF) of healthy controls and those with relapsing remitting multiple sclerosis (RRMS) (Orefice et al., 2016). Both pregnenolone (PREG) and dehydroepiandrosterone (DHEA) are significantly elevated in the CSF of MS patients of both sexes (Orefice et al., 2016). Furthermore, it was found that these changes were regulated by disease state (Orefice et al., 2016). Disease state dependent changes in NAS levels have been seen in an animal model of MS, experimental autoimmune encephalomyelitis (EAE; (Caruso et al., 2010; Giatti et al., 2010)). It was found that in the acute stage of the EAE, spinal cord levels of progesterone (PROG) and isopregnanolone (ISO) are decreased, but at the chronic stage, there is no change in spinal cord levels of these NASs (Caruso et al., 2010; Giatti et al., 2010). These studies suggest that different NASs might have different roles in disease progression (Caruso et al., 2010; Giatti et al., 2010; Orefice et al., 2016) . This is further highlighted by studies showing different changes in the levels of NASs, such as testosterone, between RRMS and progressive

MS, with males showing decreased testosterone in progressive MS compared to RRMS (Savettieri et al., 2004).

Major sex differences in the development, disease course, and symptomology of MS have been seen both clinically and in animal models, making this an important variable to consider in pre-clinical research (Lynch et al., 2008; Rahn et al., 2014; Ortona et al., 2016; Scioli-Salter et al., 2016; Mifflin et al., 2017). Sex has also been found to be a significant factor underlying the changes in NAS levels associated with MS (Caruso et al., 2010, 2014; Giatti et al., 2010; Orefice et al., 2016). For example, in a study examining changes in NASs from the CSF of patients with MS, it was found that female patients had lower levels of ALLO compared to male patients, and that in general, there were more changes in NAS levels in females compared to males (Orefice et al., 2016). Additionally, PREG levels were found to be increased in the active disease state in males compared to stable MS, but these state-dependent differences in PREG levels were not seen in female patients (Orefice et al., 2016). Interestingly, a separate study examining CSF from male MS patients only also found as well decreases in the levels of the testosterone metabolite, dihydrotestosterone (DHT), compared to non-diseased control subjects (Caruso et al., 2014).

Sex differences in NAS levels have also been reported in the EAE model. For example, it was found that female mice with EAE had larger increases in ISO and PROG in the spinal cord compared to males with EAE. On the other hand, males with EAE had increased levels of DHT compared to females (Caruso et al., 2010). Furthermore, another study examining NAS levels in acute EAE found that NAS levels change not only in a sexually dimorphic way, but also varied between plasma and different CNS regions (cerebrum, cerebellum, and spinal cord) (Giatti et al., 2010).

We have become interested in exploring the role of NASs as potential mediators of the beneficial effects of exercise in MS. To our knowledge, no previous study has examined the role or NASs in relation to the benefits of exercise in the disease, but changes in NAS levels in response to exercise in other disease states have been observed. For example, it has been shown that ALLO levels are increased after brief, but intense exercise. These changes in ALLO levels are associated with post-exercise pain tolerance in patients with post-traumatic stress disorder who also have chronic pain (Scioli-Salter et al., 2016) .

While there are only a handful of studies examining the role of exercise on NAS levels in general, the results to date suggest that exercise may be able to modulate NASs in a beneficial

way. The present study therefore explored the relationship between an exercise therapy, voluntary wheel running, and NASs associated with EAE. Given the reported sex differences in NAS levels in EAE, we also assessed NAS levels in both male and female mice with the disease.

3.1 Materials & Methods

3.1.1 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. Male and female C57BL/6J mice were obtained from Charles River Canada at age 6-8 weeks for all studies. Mice were housed in same-sex groups of 5 in standard shoebox cages with crinkle bedding with standard enrichment on a 12:12 light/dark cycle. Mice were given free access to standard chow food and water throughout all experiments. After a two week habituation period to the housing facility, mice were given EAE using myelin oligodendrocytes glycoprotein 35–55 (MOG_{35-55}). Mice were randomly assigned to Complete Freund's Adjuvant (CFA; control) or EAE (experimental) groups for all experiments EAE induction was done by giving a subcutaneous injection of 50 µg of $MOG_{35–55}$ emulsified in CFA at a concentration of 1.5 mg/mL, followed by an interaperitoneal injection of 300ng of pertussis toxin on the day of injection and again two days later. CFA control mice did not receive MOG injections, only injections of CFA and pertussis toxin. Mice were then kept in quarantine for 72 hours after the induction of EAE as per university regulations. Weight and clinical score (graded on a four-point scale) was assessed daily. The four-point clinical scale is as follows: grade 0, normal mouse; grade 1, flaccid tail (disease onset); 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. Mice who lost greater than 50% of their baseline body weight were euthanized to prevent unnecessary pain and discomfort.

3.1.2 Wheel Running

For the hour of daily running, 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″/12.5 cm #61701) for 30 consecutive days. Distance run was recorded using a Schwinn ® 20 Function bike computer (model 04SW654C6PK) fitted to the wheels. Running mice

underwent 3 consecutive days of training prior to induction. Mice started daily running on day 4 post induction after a mandatory quarantine period. (EAE male n = 5, EAE female n=5). Non-running EAE control mice spent an hour a day in a standard cage with only bedding and a fixed running wheel to control for any environmental enrichment (EAE male n = 5, CFA male n=5, EAE female n=5, CFA female =5).

3.1.3 Gas Chromatography – Mass Spectrometry (GC-MS)

Mice were euthanized via a sodium pentobartibal (340 mg/ml) injection prior to dissection. After cardiac perfusion with 0.9% saline, brains were then dissected out fresh, flash frozen in liquid nitrogen, and stored at -80℃ until homogenization. Brains were then prepared for gas chromatography–mass spectrometry (GC-MS) analysis to measure levels of NASs. Methods used were similar to those described previously by (Ahboucha et al., 2008) with minor modifications. Briefly, protein was first precipitated using methanol and then centrifugation. Supernatants were retained and NASs isolated using solid-phase extraction with Oasis HLB cartridges (Waters). Samples were then derivatized with heptafluorobutyrylimidazole (HFBI), and the resulting derivatives were analysed by gas chromatography combined with negative ion chemical ionization mass spectrometry using an Agilent 6890 gas chromatograph coupled to a 5973N mass selective detector. Standard curves were prepared for each steroid and deuterated (d_4) pregnenolone was used an as internal standard for all samples.

3.1.4 Statistical Analysis

Statistical analysis was carried out using two-tailed t tests or two-way repeated measures ANOVAs where appropriate with Tukey's multiple comparison *post hoc* tests. All values compared were a calculation of percent control of non-running CFA animals using the following formula: (experimental raw value/average of total raw CFA value) x100. Significance was set at $p < 0.05$. All statistical analysis was performed with GraphPad Prism 6.0 software.

3. 2 Results

3.2.1 Neuroactive Steroid Synthesis

All endogenous NASs originate from cholesterol. Figure 1 outlines the metabolic pathways involved in NAS synthesis and degradation. Those NASs that were detectable in the

collected brains are outlined in red (Fig. 1). Isopregnanolone, however, was only detectable in the brains of male mice (see Fig 2D). NAS levels were measured at a chronic timepoint (30 days post-induction), meaning that all mice were at a clinical score of at least 2 or higher on average.

3.2.2 Effect of Voluntary Wheel Running on NAS levels

A significant interaction for sex and exercise was found for pregnenalone, (two-way between subject ANOVA $F_{1,15}$=5.090 P=0.0394, Fig 2A). Female mice with EAE who had daily access to a running wheel were found to have significantly increased levels of pregnenolone compared to male mice with EAE given the same access to exercise (Tukey's multiple comparisons test, q=4.156, P < 0.05, Fig. 2A). A main effect of sex was also found for levels of allopregnanolone. Male mice with EAE had significantly higher levels of allopregnanolone compared to female mice with EAE (two-way between subject ANOVA $F_{1,16}$=13.92 P=0.0018, Fig. 2B). Although levels of THDOC were detectable in both male and female mice, no significant effect of sex or exercise was found (two-way between subject ANOVA, $F_{1,16}$=1.382, P=0.2569, Fig. 2C). Isopregnanolone (ISO) was only detectable in males, but running had no effect on the levels of ISO in male mice with the disease (two tailed t-test, T_8=0.0958, Fig. 2D).

3.3 Discussion

The present study explored changes in the levels of NASs in the brains of male and female mice with EAE given access to a running wheel for 1hr/day. We found that EAE caused significant changes in levels of NASs, specifically ALLO, ISO, and PREG. Furthermore, levels of PREG were found to be significantly greater in female mice with EAE that were given daily access to a running wheel.

In the current study, we observed a significant increase in ALLO levels in male mice with EAE. This is similar to a clinical report that described the levels of ALLO in male MS patients [4]. Compared to female MS patients whose levels of ALLO in the CSF were significantly reduced, the CSF from males with MS had stable levels of ALLO (Orefice et al., 2016). It should be noted however that a recent study examining levels of ALLO from the white matter of brains of patients with MS found decreased levels of ALLO (Noorbakhsh et al., 2011). However, this report did not specifically examine sex differences or measure CSF levels, possibly accounting for the discrepancy between these two studies. However, it is generally thought that ALLO is neuroprotective, and especially so in MS (see (Tuem and Atey, 2017) for a review). Thus, the

increased levels of ALLO in male mice with EAE observed here may reflect a neuroprotective mechanism in males during the chronic stages of EAE. This is further supported by the trend towards increased ALLO levels in the running cohort of male mice with EAE as we have previously shown that chronic running in males with EAE reduces clinical signs of the disease (Mifflin et al., 2017). Additionally, it has been reported that administration of ALLO or ALLO ligands to mice post-EAE induction reduces neuroinflammation and disease severity (Wang et al., 2002; Daugherty et al., 2013).

Interestingly, we found that ISO was only detectable in the male mice and these levels were independent of the running condition. This is in contrast to other studies examining sex differences in NASs in EAE. It was previously reported that ISO levels were higher in females with EAE in the cerebral cortex (Caruso et al., 2010; Giatti et al., 2010). While the results from (Caruso et al., 2010) were also from chronic EAE animals, it is important to note that their study was done in dark-agouti rats and not the C57BL/6 strain of mouse used in the present study. Importantly, a study examining changes in ISO in the CSF of patients with MS also found increased levels of ISO in males with RRMS (Caruso et al., 2014). ISO is an isomer of ALLO, and can antagonize the effect of ALLO on the GABA-A receptor, despite not binding directly to it (Wang et al., 2002; Melcangi et al., 2011). The absence of ISO in our cohorts of female mice may be a response of the low levels of ALLO combined with the high levels of PREG we have detected in these mice. A similar disequilibrium of lower levels of ISO/ALLO combined with high levels of PREG has been reported in cases of clinical depression (Melcangi et al., 2011; Schüle et al., 2011). Interestingly, it is thought that some antidepressants exert their beneficial effects by abolishing this disequilibrium (Melcangi et al., 2011; Schüle et al., 2011). A similar pattern of disequilibrium could be occurring here. However more research would be needed to explore exactly how this would impact the disease state or possible secondary symptoms such as depression or anxiety associated with MS.

The only NAS that was significantly changed with the voluntary running treatment was PREG. We found that female mice given access to the running wheels had significantly increased levels of PREG compared to male EAE mice who ran. While running did significantly elevate PREG levels in females, other studies have found that females with EAE often have higher levels of PREG in the cortex compared to males (Caruso et al., 2010; Giatti et al., 2010). To date, no study has explored the effects of exercise on PREG in MS and unfortunately there

are no other studies examining exercise and PREG in other disease states. However, it is important to note that in the clinical study examining CSF levels of NAS, increased levels of PREG were associated with active disease state in females and the presence of gadolinium enhancing lesions in male patients (Orefice et al., 2016). This suggests that increases in PREG may be promoting disease progression and/or severity. This potential effect of PREG is further supported by the fact that a previous study from our lab has shown that chronic voluntary wheel running does not improve disease scores in female mice, and may actually promote disease progression (Mifflin et al., 2017). Additionally, the decrease in PREG in males with running may also contribute to the beneficial effects of voluntary wheel running in males with chronic EAE that we have reported (Mifflin et al., 2017).

3.4 Conclusions

Overall, we find that there are significant sex differences in the brain levels of NASs in mice with chronic EAE. Furthermore, we show that daily voluntary wheel running significantly altered levels of PREG in a sex-dependent way. This may be related to some of the chronic effects of running in male and female mice. This study also highlights the importance of considering sex as a variable in MS research, especially when looking for therapeutic targets or potential biomarkers.

3.5 Figures

Figure 1.

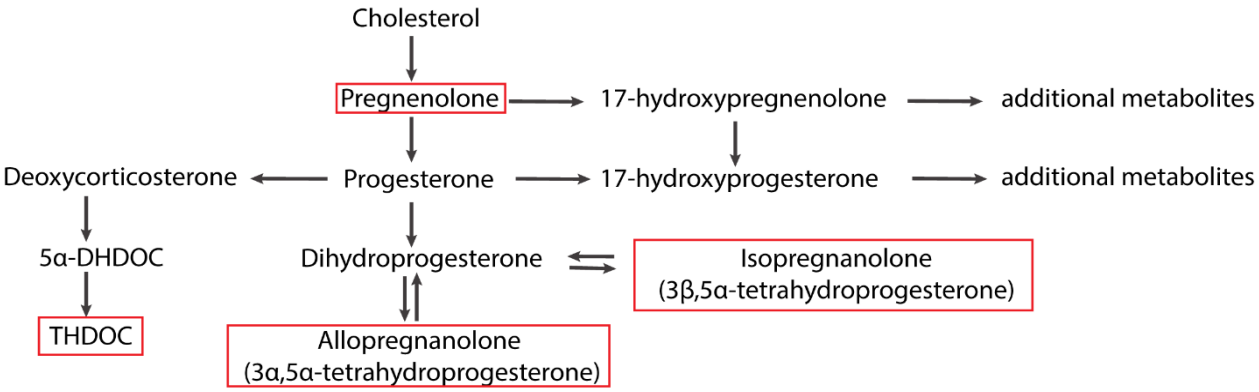

Figure 3.1 *Synthesis pathways of neuroactive steroids.*
Cholesterol is the precursor of neuroactive steroids. The flowchart outlines the various pathways in synthesis of neuroactive steroids possible from cholesterol. Those steroids reliably detected in the present study are outlined in red. *THDOC= 3α,5α-tetrahydrodeoxycorticosterone, 5α-DHDOC= 5α-dihydrodeoxycorticosterone.*

Figure 2

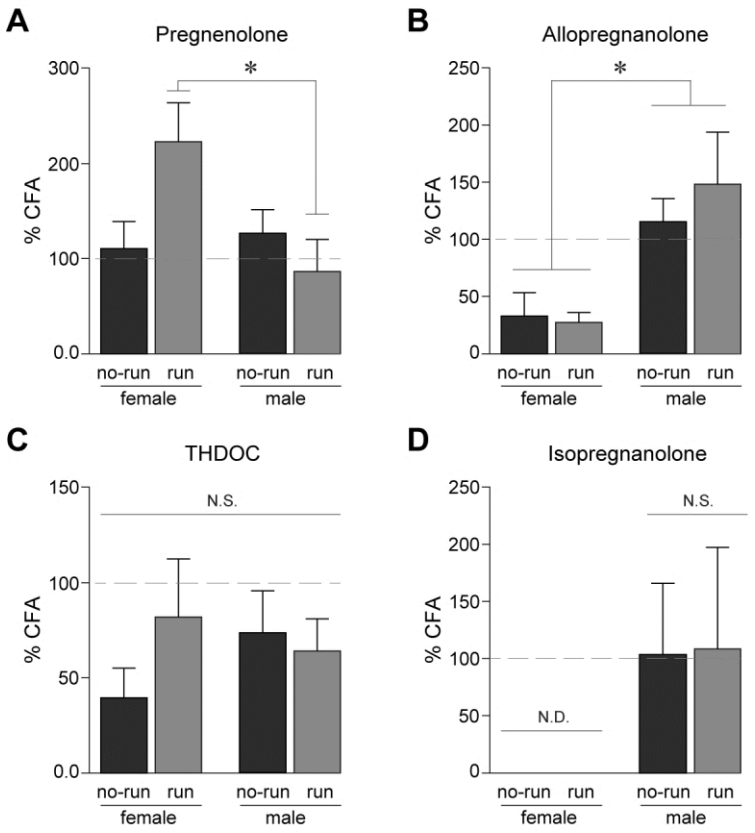

Figure 3.2. *Effect of sex and exercise on neuroactive steroid levels*.
Pregnenolone, allopregnanolone, THDOC and isopregnanolone were detectable in mouse brains. Data are expressed as a percentage of control non-running CFA mice whose average has been set to 100% as indicated by the red dashed line (A-D). Pregnenolone levels were found to be significantly increased in female EAE mice who ran compared to male EAE mice who ran (A). Alloprenanolone levels were found to be increased in male EAE mice, and especially in those who ran compared to female EAE mice (B). No significant differences were seen for THDOC in any group (C). Isopregnanolone was only detectable in male mice and no differences were seen with treatment (D). All data are expressed as mean % change from CFA ± SEM. Two-way between subject ANOVA with appropriate post-hoc analysis, *p<0.05, *THDOC= 3α,5α-tetrahydrodeoxycorticosterone, N.S.= not significant, N.D.= not detectable.* Sample size of n=5 per group.

3.6 References

D. Caruso, M. Melis, G. Fenu, S. Giatti, S. Romano, M. Grimoldi, D. Crippa, M.G. Marrosu, G. Cavaletti, R.C. Melcangi, Neuroactive steroid levels in plasma and cerebrospinal fluid of male multiple sclerosis patients, J. Neurochem. 130 (2014) 591–597. doi:10.1111/jnc.12745.

S. Giatti, G. D'Intino, O. Maschi, M. Pesaresi, L.-M. Garcia-Segura, L. Calza, D. Caruso, R.C. Melcangi, Acute experimental autoimmune encephalomyelitis induces sex dimorphic changes in neuroactive steroid levels, Neurochem. Int. 56 (2010) 118–127. doi:10.1016/j.neuint.2009.09.009.

R.H. Purdy, A.L. Morrow, P.H. Moore, S.M. Paul, Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain., Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 4553–7. http://www.ncbi.nlm.nih.gov/pubmed/1852011 (accessed April 13, 2018).

N. Orefice, A. Carotenuto, G. Mangone, B. Bues, R. Rehm, I. Cerillo, F. Saccà, A. Calignano, G. Orefice, Assessment of neuroactive steroids in cerebrospinal fluid comparing acute relapse and stable disease in relapsing-remitting multiple sclerosis, J. Steroid Biochem. Mol. Biol. 159 (2016) 1–7. doi:10.1016/J.JSBMB.2016.02.012.

G. Savettieri, D. Messina, V. Andreoli, S. Bonavita, C. Caltagirone, R. Cittadella, D. Farina, M.C. Fazio, P. Girlanda, F. Le Pira, M. Liguori, A. Lugaresi, U. Nocentini, A. Reggio, G. Salemi, G. Tedeschi, M. Trojano, P. Valentino, A. Quattrone, Gender-related effect of clinical and genetic variables on the cognitive impairment in multiple sclerosis, J. Neurol. 251 (2004) 1208–1214. doi:10.1007/s00415-004-0508-y.

D. Caruso, G. D'Intino, S. Giatti, O. Maschi, M. Pesaresi, D. Calabrese, L.-M. Garcia-Segura, L. Calza, R.C. Melcangi, Sex-dimorphic changes in neuroactive steroid levels after chronic experimental autoimmune encephalomyelitis, J. Neurochem. 114 (2010) 921–932. doi:10.1111/j.1471-4159.2010.06825.x.

K.B. Tuem, T.M. Atey, Neuroactive steroids: Receptor interactions and responses, Front. Neurol. 8 (2017) 442. doi:10.3389/fneur.2017.00442.

K.A. Mifflin, E. Frieser, C. Benson, G. Baker, B.J. Kerr, Voluntary wheel running differentially affects disease outcomes in male and female mice with experimental autoimmune encephalomyelitis, J. Neuroimmunol. 305 (2017) 135-144. doi:10.1016/j.jneuroim.2017.02.005.

E. Ortona, M. Pierdominici, A. Maselli, C. Veroni, F. Aloisi, Y. Shoenfeld, Sex-based differences in autoimmune diseases., Ann. Ist. Super. Sanita. 52 (2016) 205–12. doi:10.4415/ANN_16_02_12.

E.J. Rahn, T. Iannitti, R.R. Donahue, B.K. Taylor, Sex differences in a mouse model of multiple sclerosis: neuropathic pain behavior in females but not males and protection from neurological deficits during proestrus., Biol. Sex Differ. 5 (2014) 4. doi:10.1186/2042-6410-5-4.

J.L. Lynch, N.J. Gallus, M.E. Ericson, A.J. Beitz, Analysis of nociception, sex and peripheral nerve innervation in the TMEV animal model of multiple sclerosis., Pain. 136 (2008) 293–304. doi:10.1016/j.pain.2007.07.007.

E. Scioli-Salter, D.E. Forman, J.D. Otis, C. Tun, K. Allsup, C.E. Marx, R.L. Hauger, J.C. Shipherd, D. Higgins, A. Tyzik, A.M. Rasmusson, Potential neurobiological benefits of exercise in chronic pain and posttraumatic stress disorder: Pilot study., J. Rehabil. Res. Dev. 53 (2016) 95–106. doi:10.1682/JRRD.2014.10.0267.

S. Ahboucha, R. F. Butterworth, G. Pomier-Layrargues, C. Vincent, Z. Hassoun, G. B. Baker, Neuroactive steroids and fatigue severity in patients with primary biliary cirrhosis and hepatitis C, Neurogastroenterol. Motil. 20 (2008) 671–679. doi:10.1111/j.1365-2982.2007.01080.x.

F. Noorbakhsh, K.K. Ellestad, F. Maingat, K.G. Warren, M.H. Han, L. Steinman, G.B. Baker, C. Power, Impaired neurosteroid synthesis in multiple sclerosis, Brain. 134 (2011) 2703–2721. doi:10.1093/brain/awr200.

F. Noorbakhsh, G.B. Baker, C. Power, Allopregnanolone and neuroinflammation: a focus on multiple sclerosis., Front. Cell. Neurosci. 8 (2014) 134. doi:10.3389/fncel.2014.00134.

D.J. Daugherty, V. Selvaraj, O. V. Chechneva, X.-B. Liu, D.E. Pleasure, W. Deng, A TSPO ligand is protective in a mouse model of multiple sclerosis, EMBO Mol. Med. 5 (2013) 891–903. doi:10.1002/emmm.201202124.

M. Wang, Y. He, L.N. Eisenman, C. Fields, C.-M. Zeng, J. Mathews, A. Benz, T. Fu, E. Zorumski, J.H. Steinbach, D.F. Covey, C.F. Zorumski, S. Mennerick, 3beta -hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists., J. Neurosci. 22 (2002) 3366–75. doi:20026342.

R.C. Melcangi, G. Panzica, L.M. Garcia-Segura, Neuroactive steroids: focus on human brain, Neuroscience. 191 (2011) 1–5. doi:10.1016/j.neuroscience.2011.06.024.

C. Schüle, D. Eser, T.C. Baghai, C. Nothdurfter, J.S. Kessler, R. Rupprecht, Neuroactive steroids in affective disorders: target for novel antidepressant or anxiolytic drugs?, Neuroscience. 191 (2011) 55–77. doi:10.1016/J.Neuroscience.2011.03.025.

S. Giatti, G. D'Intino, O. Maschi, M. Pesaresi, L.-M. Garcia-Segura, L. Calza, D. Caruso, R.C. Melcangi, Acute experimental autoimmune encephalomyelitis induces sex dimorphic changes in neuroactive steroid levels, Neurochem. Int. 56 (2010) 118–127. doi:10.1016/j.neuint.2009.09.009.

Chapter 4: Voluntary Wheel Running Reveals Sex Specific Nociceptive Factors in Murine Experimental Autoimmune Encephalomyelitis

4.0 Introduction

Multiple Sclerosis (MS) is a chronic, neurodegenerative, demyelinating autoimmune disease of the central nervous system. Approximately 2-3 million people are thought to live with MS globally, making this disease a worldwide health issue (Thompson et al., 2018). The primary symptom of MS is increasing motor dysfunction over time. However, in addition to motor dysfunction, many patients experience a host of secondary symptoms including cognitive disturbances, bladder dysfunction, fatigue and chronic pain (Crabtree-Hartman, 2018). While there are several disease-modifying therapies for MS, there are few therapies that directly address these secondary symptoms, especially chronic pain. Developing better strategies to manage chronic pain in MS is of clinical importance as approximately 50% of patients with MS will develop chronic pain at some point in their disease course and this can have a significant impact on the quality of life of these patients (Kalia and OConnor, 2005; O'Connor et al., 2008; Solaro and Messmer Uccelli, 2011; Murphy et al., 2017).

Exercise has been shown both clinically and in multiple animal models of MS to have beneficial effects on disease progression (Le Page et al., 1994, 1996; Rossi et al., 2009; Patel and White, 2013; Bernardes et al., 2013, 2016, Klaren et al., 2014, 2016; Benson et al., 2015; Pryor et al., 2015; Bernardes and Oliveira, 2017; Mifflin et al., 2017; Souza et al., 2017; Kim and Sung, 2017; Reynolds et al., 2018; Venasse et al., 2018; Einstein et al., 2018). In the experimental autoimmune encephalomyelitis (EAE) model of MS, exercise has been shown to improve some secondary symptoms such as chronic pain, and cognitive dysfunction (Benson et al., 2015; Kim and Sung, 2017). While these studies demonstrate the beneficial effects of exercise on the symptoms of MS/EAE and its progression, only one study has directly explored the role of sex in the benefits of exercise (Mifflin et al., 2017).

Given the strong sex differences described in both clinical and animal studies exploring the pathobiology of MS/EAE, sex is an important variable to consider in MS research. For example, it is well known that there is a higher prevalence of MS in the females and this number is increasing (Orton et al., 2006; Thompson et al., 2018). While it is established that MS affects

females more than males, the risk of long-term disability is higher in male patients with the disease (Shirani et al., 2012; Voskuhl et al., 2018). In addition, several studies have demonstrated significant sex differences in the immune response in the EAE model (Dunn et al., 2010, 2015, Zhang et al., 2012a, 2015).

The present study was designed to explore whether the beneficial effects of voluntary wheel running on nociceptive behaviour in female mice with EAE could be extended to male mice with the disease (Benson et al., 2015). Surprisingly, we find that male mice with EAE who are given access to a running wheel for one hour per day did not show improvements in nociceptive behaviour. Here we describe assessments of various peripheral immune factors, inflammation in the CNS and sensory neuron responses to depolarizing stimuli between the sexes in our EAE-wheel running paradigm. We describe significant differences in many of these parameters, thus highlighting the importance of considering sex when exploring potential therapeutic interventions for MS.

4.1 Materials & Methods

4.1.1 EAE Induction & 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. Male and female C57/Bl6J mice were ordered from Charles River Canada at age 6-8 weeks. After two weeks of habituation to the housing facility, handling and baseline testing, mice were induced with EAE. EAE was generated with myelin oligodendrocytes glycoprotein (MOG_{35-55}; Peptide Synthesis Facility, University of Calgary, n=24). Subcutaneous (50µg) injections of $MOG_{35–55}$ emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1.5 mg/mL and an interaperitoneal injection of 300ng of pertussis toxin on the day of injection and two days later were given to mice to induce EAE. CFA control mice did not receive MOG injections, only injections of CFA and pertussis toxin (n=12). Naïve mice did not receive any MOG, CFA, or pertussis toxin injections (males, n=4; females, n=4). As per university regulations, mice were kept in quarantine for 72 hours after the induction of EAE. After release from quarantine, mice were assessed daily and weight and clinical scores were recorded. The clinical signs of EAE were graded on a four-point scale: grade 0, normal mouse; grade 1, flaccid tail (disease onset); 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 Daily Voluntary Wheel Running Paradigm

Mice were given access to a cage with a running wheel for one hour a day for starting after the mandatory quarantine period (day 4 post-induction) until disease onset (EAE male n=6, EAE female n=6). Outside of the hour of daily running, all mice were conventionally housed, 5 animals per cage, with free access to food and water. For the hour of daily running, 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″/12.5 cm #61701). Distance traveled was recorded using a Schwinn ® 20 Function bike computer (model 04SW654C6PK) fitted to the running wheel. All running group animals underwent training one week prior to the induction of EAE. During this training period, each animal in the running group was given access to the running cage for 1 hour per day for 3 consecutive days to habituate to the running wheel and to gather baseline running data. Non-running EAE control mice were transferred in a standard cage with only bedding and a fixed running wheel for one hour a day to control for any environmental enrichment (EAE male n = 6, CFA male n=6, EAE female n=6, CFA female =6). All animals were placed within the same cage with either the running wheel/fixed wheel on consecutive days over the course of the study.

4.1.3 Von Frey Hair Assessment

To assess mechanical hypersensitivity at disease onset the von Frey hair assay was used. Mice were placed in transparent plexiglass boxes on an elevated mesh wire platform to allow access to the plantar hindpaw. Mice were allowed to habituate to these plexiglass boxes for ~15minutes prior to the start of testing. After the habituation period, the plantar surface of each hindpaw was stimulated 5 times each with an ascending series of weighted von Frey monofilaments (0.16-2.0g). Behavioural responses where then observed and recorded by an experimenter blind to the conditions. Nociceptive behaviours were considered to be either lifting/guarding, rapid shaking, and/or licking of the hindpaw. If the bending force of the filaments elicited these types of behavior in 60% of more of the trails, it was considered the mechanical withdraw threshold for the mouse. Von Frey testing was conducted at baseline and at disease onset. Left and right paw

thresholds were averaged for analysis and compared to baseline levels. Baseline thresholds were assessed over 3 three testing periods in the week prior to EAE induction and averaged together.

4.1.4 RotaRod

To assess gross locomotor ability a fixed rotarod assay was used. This ensured that any motor impairment did not interfere with mechanical sensitivity testing. Mice were first trained on a 12-rpm fixed speed rotarod until they could stay on for 3 minutes in the week preceding EAE induction. On the day of disease onset, mice underwent fixed rotarod testing where they were placed on the 12-rpm speed rotarod for three, 1-minute trails. Time spent on the accelerating rotarod was averaged across the three trials.

4.1.5 Western Blotting

Mice from all experiments were euthanized via a euthansol injection and transcardiac perfusion with saline after testing. Lumbar spinal cord sections were dissected fresh and flash frozen in liquid nitrogen. The tissue was then used to prepare samples for western blot analysis for markers of spinal cord inflammation. Briefly, frozen whole spinal cord samples were homogenized in RIPA buffer (100µL NP40, 1.5mL 10% SDS, 1.5 mL 1M NaCl, 1.0mL 0.1M phosphate buffer, 40µL 0.5M EDTA, 4460µL 1x PBS, 400µL Protease Inhibitor, 1000µL PhosSTOP). Samples were then run for 1.5 hours at 100V using an SDS-page electrophoresis system from BioRAD and run on MINI-PROTEAN precast gradient 4-20% gels from BioRAD. After a 30-minute transfer at 0.35A at room temperature, the REVERT^{TM} total protein analysis kit was used to determine total protein as a loading control for analysis using a Li-COR Odyssey imaging system. After total protein assessment, polyvinylidene difluoride (PVDF) membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline solution (TBS) and then incubated with primary antibody diluted in 1% BSA overnight at 4°C (Rabbit α pP65 1:500 Millipore Cat# AB3375, RRID:AB_240665). After incubation, membranes were washed twice in TBS-Tween and one time in 1xTBS. Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 45 minutes at room temperature (goat α rabbit HRP, 1:25,000) and visualized using an ECL system (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer). All blots were quantified using Image Studio Lite software and 2 log ratios were calculated to compare experimental groups to control non-running CFA groups for each sex.

4.1.6 Oxidative Stress Assay

The introduction of carbonyl groups into proteins is due to side-chain reactions with oxygen free radicals and other reactive species. The measurement of carbonyl groups can therefore provide a measure of total protein oxidation. To determine differences in levels of oxidative stress between running and non-running male and female mice at disease onset, we used the Abcam Oxidized Protein Western Blot Detection Kit (Cat #:ab178020). This kit is designed to measure the free radical modification of side chains into carbonyl groups. Spinal cord tissue samples homogenized in RIPA buffer (see above) were used for analysis. Kit instructions were followed, but briefly, samples were denatured by adding 12% SDS to each aliquot, and carbonyl groups in the samples were derivatized by adding 1X DNPH Solution (or 1X Derivatization Control Solution for negative controls) to each aliquot. Samples were then incubated at room temperature for 15 minutes, then neutralized with the supplied solution to yield treated samples and controls. 5 µg/µL aliquots of derivatized sample and the corresponding control, along with a molecular weight ladder and a 10µL DNP-BSA standard, were loaded onto MINI-PROTEAN precast 4-20% SDS-PAGE gels from BioRad. Gels were run at 150V for one hour with a BioRad electrophoresis system, and transferred at 0.35A for one hour at 4ºC onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in PBS-Tween for one hour at room temperature, then incubated in DNP primary antibody (Abcam Rb α-DNP, 1:5000, provided in the kit) overnight at 4ºC. Membranes were then washed three times in 1X PBS-T for 5 minutes each, then incubated in HRP-conjugated secondary antibody (Gt α-Rb, 1:5000) for one hour at room temperature. Membranes were then washed an additional three times in 1X PBS-T for 5 minutes each, then rinsed in 1X PBS. Imaging was performed using a chemiluminescence (ECL) system and quantified with ImageStudioLite software.

4.1.7 Splenocyte Cultures, Proliferation Assay, and Cytokine Analysis

Mice were euthanized at disease onset via a euthansol injection and spleens were quickly removed and placed in 5ml media (RPMI 1640 supplemented with l2 mM L-glutamine, 1MM sodium pyruvate, 0.1mM nonessential amino acids, 100 U/ml penicillin, 0.1mg/ml streptomycin, 0.5 µM 2-mercaptoethanol and 10% heat inactivated FCS) on ice. Spleens were then

homongenized and filtered through a 70 micron mesh filter with an additional 5ml media. The resulting cell suspension was then centrifuged (2500RPM for 5 minutes). The supernatant was then removed, the pellet was resuspended in ACK lysis buffer for 2 minutes to eliminate red blood cells, then 10ml RPMI was added to inactivate the buffer. The resulting cell suspension was then centrifuged two more times to wash the ACK lysis buffer from the cells. Cells were then counted with a hemocytometer and plated at 500,000 cells per well. Wells were then stimulated with various concentrations of the MOG_{35-55} peptide in media (triplicate wells for each condition; 10µg/ml, 5µg/ml, 2.5µg/ml, 0µg/ml) and incubated for 48 or 72 hours. After incubation, plates were immediately frozen at -80℃ until supernatant was collected for cytokine analysis. Plates were then thawed and then centrifuged (2500 RPM for 5 minutes) and the resulting supernatant was collected for cytokine analysis. Cytokines were assessed using kits (Invitrogen Uncoated ELISA kits, Thermo Fisher Scientific) and were assessed at the time of peak production for each cytokine (48hrs- INFγ, IL-17a, 72hrs- TNFα). An additional plate, which was incubated for 48 hours, was prepared for the proliferation assay. To measure proliferation rate, separate cultures were incubated for 48 hours with MOG_{35-55} stimulation and then pulsed with [^{3}H]thymidine (1µCi/well). After 20h, cells were harvested onto filter paper and the counts per minute of incorporated [^{3}H] were then read using a scintillation counter.

4.1.8 Dissociated Dorsal Root Ganglia Cultures

Prior to dorsal root ganglia (DRG) culturing, 15mm coverslips (Fisher, cat no. 12-545-83) were individually placed in a 12-well plate and were coated with 25 µg/mL poly-D lysine (PDL; Sigma, cat no. P6407) overnight at 4℃. The following morning, PDL was removed and the coverslips were washed five times with tissue culture water (Hyclone, cat no. SH30529.02). The coverslips were then allowed to dry in a tissue culture hood. Just prior to dissection, the coverslips were coated with laminin (Invitrogen, cat no. 23017-015) diluted in modified Hank's Balanced Salt Solution (mHBSS; Hyclone, cat no. SH30031.02) to a final concentration of 10µg/mL. The coverslips were then placed in an incubator (37℃ and 5% CO_2) until needed.

After the removal of spleens for splenocyte cultures, mice were intracardially perfused with ice-cold 0.9% saline. The lumbar 4,5, and 6 DRGs were dissected, placed in Hank's Balanced Salt Solution (HBSS; Hyclone cat no. SH30030.02) and transferred to a sterile conical tube. The DRGs were then washed three times with HBSS (1 mL/wash). After the final wash, the

HBSS was replaced with 3 mL of HBSS containing 2 mg/mL collagenase type IV (Worthington, cat no. LS002132) and 0.1 mg/mL DNase (Worthington, cat no. LS004188). The DRG-containing conical tube was then placed in a water bath heated to 37℃ with gentle shaking. After 45 minutes in the enzyme solution, the tissue was gently triturated with a p1000 pipette 40 - 50 times and the conical tube was returned to the water bath for an additional 10-15 minutes. The tissue was then triturated again with a p1000 pipette (20-30 times) followed by a p200 pipette (20-30 times). Once the tissue was digested, the conical tube was centrifuged (5 minutes at 500 relative centrifugal field (RCF)) and 2 mL of the enzyme solution was removed. The cell pellet was then resuspended in the remaining enzyme solution, which was subsequently passed through a 70 μM filter and then placed onto 2 mL of 150 mg/mL bovine serum albumin (BSA; dissolved in HBSS). The conical tube was centrifuged again (5 minutes at 900 RCF) and the supernatant was aspirated. The cell pellet was then resuspended in 2mL of DMEM/F12 (Gibco, cat no. 10565018) and centrifuged again (5 minutes at 500 RCF). The DMEM/F12 was aspirated and the pellet was resuspended in 300 μL of cell media (DMEM/F12 containing 1% N2 (Gibco, cat no. 17502048) and 1% penicillin/streptomycin (Gibco, cat no. 1570063)). The cell suspension was then spread across three coverslips which were then transferred to an incubator (37℃, 5% CO_2). One hour after plating, wells with coverslips were flooded with 1 mL of warm cell media.

4.1.9 Ca^{2+} Imaging of Dorsal Root Ganglia Cultures

Cytosolic calcium imaging was carried out 24 h after the dissociated DRG neurons were placed on the coverslips. Prior to imaging, each coverslip was incubated at 37℃ and 5% CO_2 in HBSS (Gibco, 14025092) containing 10 μM Fluo-4 AM (Invitrogen, F14201) for 30 minutes. After the incubation period, each coverslip was then transferred to a chamber that was continually perfused (4 mL/min) with a physiological solution (PS; 120 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 20 mM glucose). After 5-10-minute perfusion, a 5-minute recording was initiated which consisted of: 30 second perfusion with PS, 30 second perfusion with PS containing 20 mM caffeine, 4 minute perfusion with PS. A second recording was initiated 1-2 minutes after the first recording and consisted of: 30 second perfusion with PS, 30second perfusion with PS containing 30mM KCl, 4-minute perfusion with PS. The imaging was carried out on an Olympus FV1000 confocal laser scanning microscope. Images were captured at 1 frame/second and all image acquisition settings were kept constant throughout the experiment.

Ca^{2+} imaging data were extracted using Olympus FV10-ASW software. The first 30 seconds of recording was treated as baseline and averaged. The rest of the recording was divided by that average to obtain at a ratio of change from the baseline (Fluo-4/F). The diameter of a cell was estimated using a modified scale bar on the analysis software. Only fully dissociated cells were used for analysis. The area under the curve was obtained for each cell's individual response pattern using GraphPad Prism 6.

4.1.10 Conditioned Media DRG Culture and Calcium Imaging Experiments

To determine if circulating peripheral cytokines could alter DRG activity, DRGs were cultured from naïve mice as described above, with STEMzyme I (Worthington, cat no. LS004106) replacing collagenase IV. After dissociation, these cells exposed to media from the previously described spleneocyte cultures. Briefly, after dissociated DRGs were placed on coated glass coverslips, they were incubated with conditioned splenocyte media from the 10 µg/ml MOG_{35-55} stimulated conditions for at least 4 hours prior to calcium imaging. Calcium imaging was conducted as described and analyzed as above.

4.1.11 Experimental Design and Statistical Analysis section

Statistical analysis was carried out using two tailed unpaired t tests, one-way ANOVAs or two-way ANOVAs with appropriate *post hoc* tests. For non-parametric data, an ANOVA on ranks was used or a Mann-Whitey U test, where appropriate. Significance was set at $p < 0.05$. All statistical analysis was performed with GraphPad Prism 6.

4.2 Results

4.2.1 The Effect of Voluntary Wheel Running on Nociceptive Behaviour

Starting at day 4 post-induction, mice were either given access to a running wheel for 1 hour per day until disease onset, or were given access to a cage with a locked wheel as a non-running control. At the first clinical sign of EAE (a flaccid tail), hind paw mechanical hypersensitivity was assessed using von Frey monofilaments. Mice immunized only with the CFA adjuvant were used as controls for EAE. We observed no significant change in the mechanical sensitivity of CFA control mice of either sex (females $t(4)=1.369$, $p=0.2427$; Fig. 1ai, males $t(4)= 0.5096$, $p=0.6372$; Fig. 1bi). As expected, both male and female mice with EAE given access only to locked running wheels developed hind paw mechanical hypersensitivity (females $t(3)=35.00$,

p=0.0001; Fig 1.aii, males t(3)=3.390, p=0.0428; Fig. 1bii). Having access to a functioning running wheel did however, prevent mechanical hypersensitivity from developing in female mice with EAE (t(4)=0.2968, p=0.7814; Fig. 1aiii). Male mice with EAE given the same access to a running wheel for 1 hour per day continued to exhibit tactile hypersensitivity (t(4)=3.469, p=0.0256; Fig. 1biii).

The sex differences in mechanical hypersensitivity did not appear to be due to differences in running parameters. Both male and female mice ran an equivalent total distance over the testing period (t(10)=1.206, p=0.2557, Fig. 2a). There was also no difference in the day to onset between running and non-running mice of either sex (females t(9)=0.8243, p=0.4311, males t(8)=0.2227; Fig. 2b). Furthermore, there were no differences in gross locomotor function between groups at this early stage of the disease as measured by the rotarod test, (one way ANOVA F(2,12)=0.2996, p=0.7465, Fig. 2ci) or males (one way ANOVA F(2,11)=0.2286, p=0.7993, Fig. 2cii). Taken together, these observations suggest that the differences in the response to wheel running on mechanical hypersensitivity were not due to effects on locomotor ability or running parameters.

4.2.2 The Effect of Voluntary Wheel Running on Peripheral Immune Responses

Splenocyte cultures were used to assess proliferation and peripheral immune responses to MOG_{35-55} antigen between the sexes in the different conditions. Analysis of splenocyte proliferation revealed that splenocytes from EAE mice from both sexes proliferated more than splenocytes from non-disease controls immunized with CFA alone (two way ANOVA, main effect of group, females $F(2,47)=17.355$, $p<0.001$, males $F(2,48)= 18.629$, $P<0.001$, Fig. 3Ai). Interestingly, proliferation was increased further in male mice with EAE in the voluntary wheel running condition compared to CFA and non-running groups (Holm-Sidak multiple comparisons, CFA vs Run $t(48)=6.100$, $p<0.001$, No Run vs Run $t(48)=2.860$, $p<0.006$, Fig. 3Aii).

Voluntary wheel running also influenced peripheral cytokine production. The levels of INFγ were increased with EAE in both female (two way ANOVA, main effect of group $F(2,48)=7.875$, $p=0.011$, Fig. 3bi) and male mice (two way ANOVA, main effect of group $F(2.47)=11.21$, $p=0.0001$; Fig. 3bii). Voluntary wheel running in male and female mice also

increased INFγ levels compared to non-running mice (Tukey's multiple comparison test, females: q(48)=5.571, P<0.05 Fig. 3bi, males: q(47)=4.546, p <0.05; Fig, 3bii).

Two cytokines associated with the development and maintenance of chronic pain were also analyzed: TNFα and IL-17a. TNFα levels were found to be elevated in non-running EAE groups in both females (two way ANOVA, main effect of group F(2,48)=4.455, P=0.0168, Tukey's multiple comparison test CFA vs NR q(48)= 4.003 p<0.05; Fig. 3ci) and males (two way ANOVA, main effect of group F(2, 28)= 17.60, P<0.0001; Tukey's multiple comparison test CFA vs NR q(48)=3.965, p<0.05; Fig. 3cii). Voluntary wheel running prevented the increase in TNFα in female mice (Tukey's multiple comparison test CFA vs Run q(48)=0.8420, P>0.05; Fig. 3ci), but interestingly, exacerbated this increase in male mice (Tukey's multiple comparison test CFA vs RUN q(48)=8.385, p<0.05 and NR vs RUN q(48)= 4.420, p<0.05, Fig. 3cii). A similar pattern was observed when we assessed the levels of IL-17a. EAE increased IL-17a levels in both non-running female (two way ANOVA main effect of group F(2,48)= 10.15, p=0.002, Tukey's multiple comparisons CFA vs NR q(48)=4.661 P<0.05, CFA vs RUN q(48)=6.094, p<0.05; Fig. 3di) and male mice (two way ANOVA main effect of group F(2,47)= 16.325, p<0.0001, Tukey's multiple comparisons CFA vs NR q(47)=4.074 P<0.05, CFA vs RUN q(47)=8.077, p<0.05; Fig. 3dii). However, this increase in IL-17a was exacerbated in male mice with EAE who had access to the running wheels (Tukey's multiple comparisons NR vs Run q(47)=3.898, P<0.05; Fig. 3dii). Overall these results suggest that the sustained nociceptive behaviour in male mice who ran may be related to an increased inflammatory peripheral immune profile.

4.2.3 The Effect of Voluntary Wheel Running on Inflammatory Events in the Spinal Cord

We next assessed the level of pP65 in the spinal cord from the different groups of mice. pP65 has been used previously as an indicator of activated NF-κB and a measure of spinal cord inflammatory signaling (Han et al., 2014; Cimpean et al., 2015). Surprisingly, having access to a running wheel increased the levels of pP65 in both male (one way ANVOVA, F(2.12)=4.086, P=0.0443 Tukey's multiple comparisons CFA vs Run q(12)= 3.945, p=0.0.403; Fig. 4a) and female mice with EAE (one way ANOVA, F(2,12)=4.354, P=0.0379, Tukey's multiple comparisons CFA vs Run q(12)= 4.125, p=0.0322; Fig .4a). As a secondary measure of spinal inflammatory processes, we also assessed the levels of oxidative stress in the spinal cord. We

measured total protein carbonylation as an indicator of oxidative stress and observed that female mice with EAE had increased amounts of carbonylated proteins in the spinal cord at disease onset (one-way ANOVA ($F(2,12)= 5.906$, $p = 0.0164$; Fig. 4b). These levels are further enhanced in female mice with EAE from the wheel running group compared to non-disease, CFA controls (Tukey's multiple comparison post-hoc test ($q(12)=4.856$, $p<0.05$; Fig. 4b). Interestingly, there is no significant difference in the levels of protein carbonylation/oxidative stress between any of the male groups of mice (one-way ANOVA test $F(2,12) = 1.895$; $p = 0.1926$; Fig. 4b). Overall, these results suggest that there are distinct inflammatory processes at the spinal level between the sexes and that wheel running can potentiate indicators of oxidative stress in the spinal cord of female mice.

.

4.2.4 Effect of Voluntary Wheel Running on DRG Hyperexcitability

Given that the wheel running paradigm was not exerting any major effects on inflammatory processes in the spinal cord, we next wanted to determine if the effects of wheel running on nociceptive sensitivity were being mediated at the level of the sensory neurons in the DRG. We therefore used calcium imaging on cultures of sensory neurons from CFA controls and mice with EAE in the different conditions and monitored the responses to depolarization with 30mM KCL. Representative traces from small (Fig. 5Ai, 5Bi) and large (Fig. 5Aiii, 5Biii) diameter neurons in the cultures demonstrate the significant differences in Ca^{2+} responses between female and male sensory neurons with EAE. There is a significant increase in the amplitude of the response to KCL depolarization from small diameter neurons from female mice with EAE who did not have access to a running wheel (one way ANOVA $F(2,395)=3.024$, $P=0.0497$, Tukey's multiple comparison test CFA vs NR $q(395)=3.335$, $p=0.0493$; Fig. 5Aii). Large diameter neurons from these mice also exhibited heightened Ca^{2+} responses to KCl depolarization (one way ANOVA $F(2,94)=4.708$, $P=0.0113$, Tukey's multiple comparison test CFA vs NR $q(94)=3.724$, $p=0.0265$, NR vs Run $q(94)=3.828$, $p=0.0218$; Fig. 5Aiv). Interestingly, there were no significant differences in Ca^{2+} transients between female non-disease, CFA controls and female EAE mice who had access to the running wheels. This was true for both small (Tukey's multiple comparison test CFA vs Run $q(395)=0.8994$, $p=0.1849$; Fig. 5Ai,ii) or large diameter DRG neurons (Tukey's multiple comparison test CFA vs Run $q(94)=0.0494$, $p=0.9993$; Fig. 5Aiii,iv).

We also observed significant changes in the Ca^{2+} responses to KCL depolarization from the sensory neurons of male mice with EAE. In contrast to female sensory neurons, we observed a slight reduction in the amplitude of the Ca^{2+} transients in small diameter neurons from male mice with EAE who did not run (Kruskal-Wallis test statistic=12.46, P=0.002, Dunn's multiple comparison test CFA vs NR mean rank diff. =44.61, p=0.0017; Fig. 5Bi,ii). More strikingly, we found that there is a dramatic increase in the amplitude of Ca^{2+} transients from large diameter neurons in male mice with EAE given daily access to a running wheel (one way ANOVA F(2,102)=3.593, p=0.0310, Tukey's multiple comparison test NR vs Run q(102)=3.628, p=0.0313; Fig. 5Biii,iv).

4.2.5 Conditioned Media from EAE mice can Affect the Excitability of DRG Neurons from Naïve Mice

To determine if the changes in DRG excitability in mice with EAE were due to the changes in peripheral cytokine levels in response to the disease or to an intrinsic property of the diseased neurons, we used the conditioned media (CM) from the splenocyte cultures of non-running and running mice with EAE of both sexes and challenged sensory neurons from the DRGs of naïve mice. Media from 0 μg/ml or 10 μg/ml MOG_{35-55} stimulation from both 48h and 72h cultures were used to stimulate naïve DRG neurons. Both the 48h and 72h time points were used to capture the different cytokine profiles that are released over these time points (see methods for details).

Small diameter neurons from naïve female mice incubated with the 48 or 72h media (10 μg) had increased Ca^{2+} responses to KCl depolarization (two way ANOVA significant interaction of treatment and media, F(2,323)=7.850 P=0.0005, Tukey's multiple comparison test 0 vs 48 q(323)= 3.682, p=0.0261, 0 vs 72 q(323)=7.335, p<0.0001, 48 vs 72 q(323)=4.852, p=0.002; Fig 6Ai, iii). We observed the largest increases with the 72h non-running media (Tukey's multiple comparison test 48 vs 72 q(323)=4.852, p=0.002; Fig 6Ai -iii). There were no changes in the Ca^{2+} responses of small diameter neurons that were treated with the CM from female running mice with EAE (Tukey's multiple comparison test 0 vs 48 q(323)=0.5474, p=0.9208, 0 vs 72 q(323)=7.335, p=0.9074, 48 vs 72 q(323)=0.0922, p=0.9977; Fig. 6Aii, Aiii). The Ca^{2+} responses of large diameter neurons from naïve female mice were also affected in response to 48h and 72h CM from non-running female mice with EAE. These effects were not

seen when the CM from running mice was used (two way ANOVA main effect of running F(2,152)=, p=11.80, p=0.0008; Fig. 6Bi, Biii).

Ca^{2+} responses of DRG neurons from naïve male mice were also affected by incubation with the CM from male mice with EAE. The 48h CM from non-running male mice with EAE increased the Ca^{2+} responses of small diameter DRG neurons (two way ANOVA, significant interaction of running and media F(2,292)= 3.029 , p<0.0499, Tukey's multiple comparison test NS 0 vs 48 q(292)=3.931, p=0.0159; Fig. 6Ci, Ciii). There was no change in Ca^{2+} responses from small diameter neurons from naïve male mice when they were incubated with the CM from running male mice with EAE (Tukey's multiple comparisons test running 0 vs 48 q(292)=1.088, p=0.7223, 0 vs 72 q(292)=1.844, p=0.3566, 48 vs 72 q(292)=2.297 p=0.8017, Fig. 6Cii, Ciii). The 72h CM from running male mice with EAE did however, significantly increase the Ca^{2+} responses of large diameter neurons (two way ANOVA, significant interaction of running and media F(2, 238)= 4.455, p=0.0126, Tukey's multiple comparison test 0 vs 72 q(238)=0.9568, p=0.0035, 48 vs 72 q(238)=4.397, p=0.0059; Fig. 6Dii, Diii). Again, these results suggest that the peripheral cytokines can directly sensitize DRG neurons.

4.3 Discussion

In recent years, there has been a number of studies describing the benefits of exercise in patients with MS. The specific cellular mechanisms of how exercise exerts these benefits however, remains poorly understood. In addition, variables such as sex may influence the effects of exercise. In the present study, we addressed how sex might influence pain in response to an exercise regimen and elucidated the mediators of these effects in an animal model of MS.

A previous study by our group examining the effect of sex on disease outcomes in response to exercise in the EAE model was the first to address this issue (Mifflin et al., 2017). We demonstrated that daily access to a running wheel reduced disease severity in male mice with EAE but not females with the disease. This is a significant sex difference that had not been previously reported (Mifflin et al., 2017). In the present study, we also find a significant effect of sex: we find that exercise modulates the tactile hypersensitivity commonly observed in mice with EAE at disease onset in a sex-specific way. This replicates the findings of Benson et al. who reported that voluntary wheel running reduces tactile hypersensitivity in female mice with EAE at disease onset (Benson et al., 2015). Surprisingly, voluntary wheel running did not alter

nociceptive hypersensitivity in male mice with EAE. To understand this behavioural difference, we first asked whether differences in running behaviour between the sexes might mediate these different responses to exercise. However, we found no differences in the average total distance run or gross locomotor function as assessed by performance on the rotorod between the different groups of mice.

We next examined whether there are sex differences in the immune response to wheel running in the disease as it is well established that there are significant sex differences in the immune response to MS/EAE and in the development of chronic pain (Dunn et al., 2007; Greenspan et al., 2007; Zhang et al., 2012a, 2015; Sorge et al., 2015). Our analysis of the proliferation and cytokine production in response to MOG_{35-55} stimulation of splenocyte cultures revealed that male mice with EAE who had access to a running wheel had a more pro-inflammatory peripheral immune profile compared to female mice with EAE who ran. We observed increased levels of TNFα and IL-17a in male mice with EAE mice given 1hr/day access to the running wheels. In contrast, female mice who ran did not have elevated levels of IL-17a and exhibited a significant reversal in TNFα levels. Changes in these two pro-inflammatory cytokines are significant as it is known that increased levels of TNFα and IL-17a are associated with chronic pain in various animal models and patient populations (Kim and Moalem-Taylor, 2011; Berta et al., 2014; Day et al., 2014; Liu et al., 2015; Yao et al., 2016; Giacoppo et al., 2017; Sun et al., 2017; Chen et al., 2018; Wen et al., 2018; Langjahr et al., 2018; Tian et al., 2018; Wang et al., 2018). These results provide evidence of a sustained and potentiated pro-inflammatory profile in male mice with EAE who ran.

Given these differences in the peripheral immune response, we next wanted to determine if wheel running or sex had an influence on the inflammatory environment of the spinal cord associated with EAE. Interestingly, we found that inflammatory signalling, as assessed by western blots of p-P65 expression, was elevated in all groups of mice with EAE and these levels were augmented in both the male and female cohorts given access to the running wheels. We also observed increased indices of oxidative stress in the spinal cords of female mice with EAE that was again potentiated by wheel running. This enhancement of NF-κB activation and oxidative stress was surprising given that previous studies have reported general reductions in spinal cord inflammatory markers with exercise (Benson et al., 2015; Pryor et al., 2015; Bernardes et al., 2016; Souza et al., 2017). It is important to note however, that these studies

were examining inflammation at more chronic time points in the disease course and not at the onset of disease (Pryor et al., 2015; Bernardes et al., 2016; Souza et al., 2017). Additionally, two recent studies found that it was changes in peripheral immunomodulation in EAE that were responsible for the benefit of exercise, rather than direct neuroprotection in the CNS (Souza et al., 2017; Einstein et al., 2018).

Changes in the excitability of peripheral sensory neurons of the DRG have been implicated in the development and maintenance of chronic pain in multiple models (see (Krames, 2015; Berta et al., 2017) for review of the role of DRG in chronic pain). Furthermore, a study by Lopez-Alvarez and colleagues found that early treadmill exercise after sciatic nerve injury prevented the development of neuropathic pain by altering the expression of chloride co-transporters in the DRG (López-Álvarez et al., 2015). While this study did not directly look at DRG hyperexcitability, it provides evidence that exercise can elicit changes in the DRG to alter nociception (López-Álvarez et al., 2015). In line with this, we found significant changes in the intracellular Ca^{2+} responses to depolarizing stimuli in the sensory neurons of mice with EAE. The Ca^{2+} responses from both small and large diameter DRG sensory neurons were increased in non-running females with EAE. Interestingly, the intracellular Ca^{2+} response after KCl depolarization was unchanged in those female mice who had access to a running wheel compared to non-diseased, CFA controls. This suggests that the reduction in nociceptive behavior we have observed in female mice with EAE given access to the running wheels is due to a reduction in the responsiveness of sensory neurons to depolarization. In contrast, we find that male mice in the wheel running cohort had increased DRG Ca^{2+} responses to KCl depolarization and sustained nociceptive behaviour. Interestingly, we did not find any significant change in the excitability of sensory neurons from male mice with EAE who did not run. In fact, we found slight but statistically significant reductions in the Ca^{2+} responses to KCl depolarization in small diameter neurons, despite those mice still demonstrating tactile hypersensitivity at disease onset. This suggests that tactile hypersensitivity in male mice with EAE may be independent of small diameter nociceptors. Given that EAE is a demyelinating disease, myelinated sensory neurons likely sustain more damage than smaller, unmyelinated ones. Furthermore, a previous study found that injury in sensory neurons in EAE is detectable prior to disease onset (Frezel et al., 2016). This study also found that the MOG induced T-cell infiltration into the DRG was from the CNS, rather than the PNS, suggesting that changes in the

male DRG may be due to changes that occur in the CNS (Frezel et al., 2016). We observed a significant enhancement of the responsiveness of large diameter sensory neurons in wheel running male mice with EAE. This may be due to an exacerbation of damage to myelinated fibers because of the increased levels of peripheral cytokines, T-cell proliferation, and spinal cord inflammation that we have noted in male mice given access to the running wheels.

To determine if these observed changes in Ca^{2+} responses to depolarizing stimuli are a response to secreted factors from inflammatory cells, we used DRG sensory neurons from naïve mice that were incubated with the CM from splenocyte cultures of running and non-running EAE mice. We predicted that the CM from splenocytes from those groups that demonstrated enhanced nociceptive behaviours would sensitize naïve neurons due to the elevated levels of pro-inflammatory cytokines in the CM. Indeed, we found that the media from both the 48 and 72h splenocyte cultures from non-running female mice increased the Ca^{2+} signal in response to KCl depolarization from small and large diameter neurons in a manner analogous to what we observed from the sensory neurons of non-running female mice with EAE. We observed no significant differences in the Ca^{2+} responses when naïve sensory neurons were incubated with the CM from splenocytes of female mice with EAE given access to running wheels. In DRG cultures from naïve male mice, we found that both small and large diameters neurons were sensitized by the CM. The greatest changes occurred in large diameter neurons incubated with the CM from 72h splenocyte cultures from the wheel running male mice with EAE. This reflects the increased Ca^{2+} responses we observed in large diameter neurons from male mice with EAE who ran 1hr/day. Interestingly, the 48h CM from non-running male mice was also able to sensitize small diameter neurons from naïve male mice. This is in contrast with the changes we observed in small diameter neurons from the DRG of non-running male mice with EAE. These differences likely come from the origin of the cultured neurons being stimulated with KCl: naïve vs EAE. As mentioned above, it is likely that the large diameter neurons from mice with EAE sustain some damage prior to disease onset, whereas this damage would not be present in the DRG cultures from naïve mice. As a result, the small diameter neurons, more traditionally involved in nociception, would be sensitized by the CM in the naïve cultures compared to the large diameter neurons.

4.4 Conclusions

The present study is the first to demonstrate a sex dependent effect of voluntary wheel running in the modulation of nociceptive behaviour in mice with EAE. We demonstrate that this sex difference is dependent on the different immunological profiles of male and female mice with the disease. Overall, male mice with EAE who had access to the running wheel have an elevated pro-inflammatory profile compared to female EAE mice who ran. The present study also demonstrates that it is changes in the responsiveness of sensory neurons in the DRG in response to peripheral cytokine levels that contribute to the observed changes in nociceptive sensitivity. Ultimately, our results highlight the importance of sex as a critical variable underlying the pathophysiological outcomes of EAE and MS.

4.5 Figures

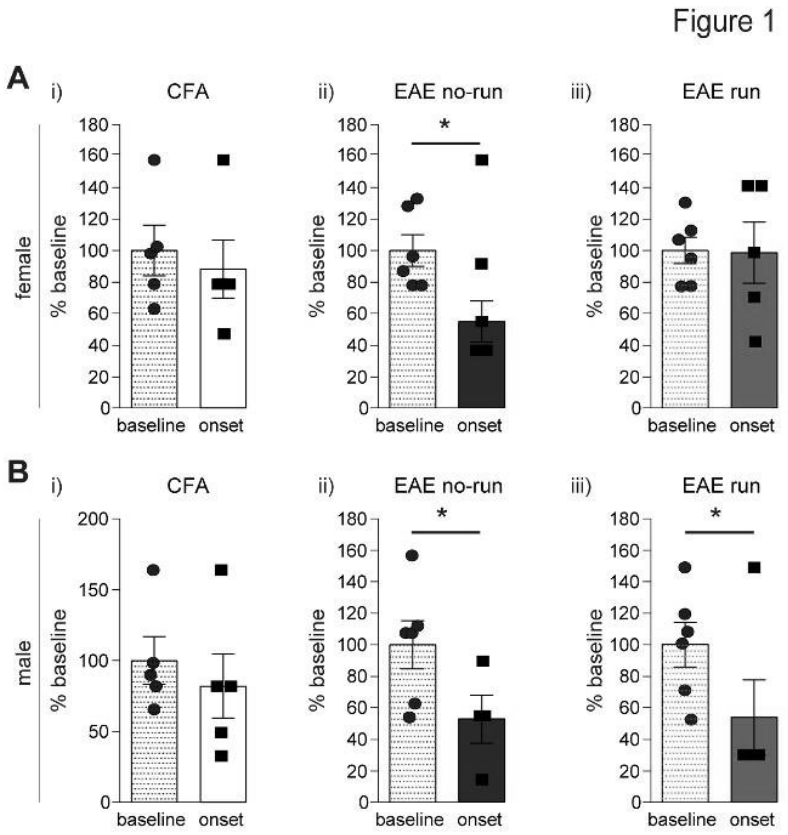

Figure 4.1. *Voluntary wheel running reduced nociceptive behaviour in female mice but not male mice with EAE.* CFA mice did not display any change in nociceptive behaviour from baseline to onset as measured by von Frey monofilaments (Ai & Bi). Both male and female mice who did not have access to a running wheel (no run mice) displayed reduced mechanical thresholds at disease onset compared to baseline (Aii & Bii). Having access to a running wheel for an hour a day did reverse the nociceptive behaviour seen in female mice (Aiii), but did not do so in male mice (Biii). Bars indicate mean ± standard error of mean (SEM). *$p < 0.05$, two tailed unpaired t-tests.

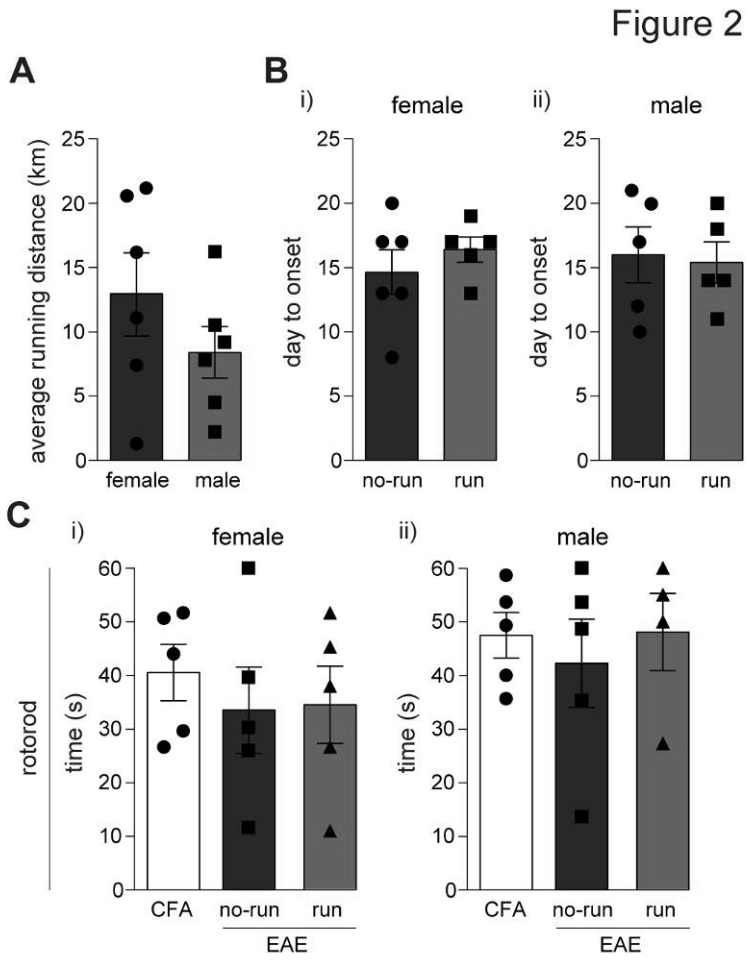

Figure 4.2. *No differences seen in running parameters seen between the sexes*. No significant difference was seen in the average distance ran between male and female mice (A). In addition, daily voluntary wheel running did not significantly delay disease onset in either sex (B). Finally, motor function was equivalent between CFA and both disease groups at the day of onset in both sexes (C). Bars indicate mean ± standard error of mean (SEM). *$p < 0.05$, two tailed unpaired t-tests (A & B) of one- way ANOVA (C).

Figure 3

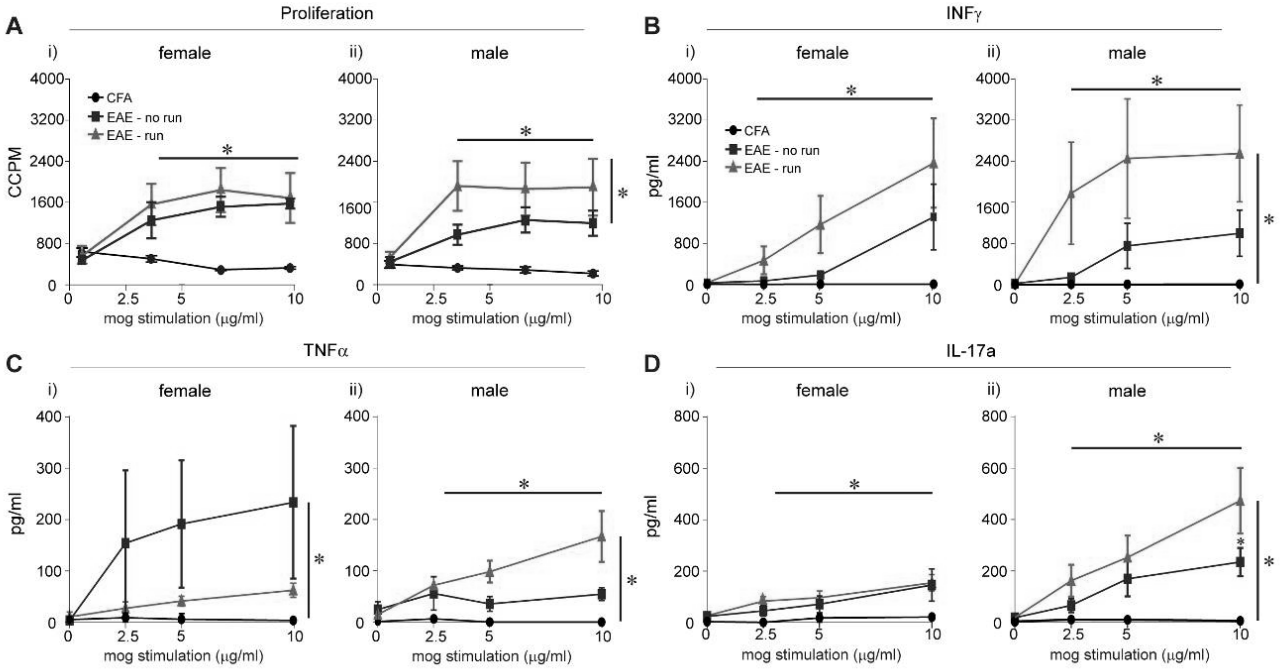

Figure 4.3. *Effect of voluntary wheel running on peripheral immunity.* Proliferation analysis revealed that splenocytes from EAE mice had increased proliferation compared to CFA animals in both sexes (A & B). Running in the male EAE mice further increased proliferation (B). Running also affected peripheral cytokine levels. Levels of INFγ were increased with EAE in both female (Bi) and male mice (Bii). Voluntary wheel running in male mice also increased INFγ compared to non-running mice (Bii). TNFα levels were found to be elevated in non-running EAE mice in both female (Ci)and male mice (Cii). Voluntary wheel running prevented this increase in TNFα levels in female mice (Ci), but exacerbated this increase in male mice who ran (Cii). EAE also increased IL-17a levels in both female (Di) and male mice (Dii). This increase in IL-17a was further increased in male mice who ran (Dii). Bars indicate mean ± standard error of mean (SEM). *$p < 0.05$, two-way ANOVAs with tukey's multiple comparisons.

Figure 4

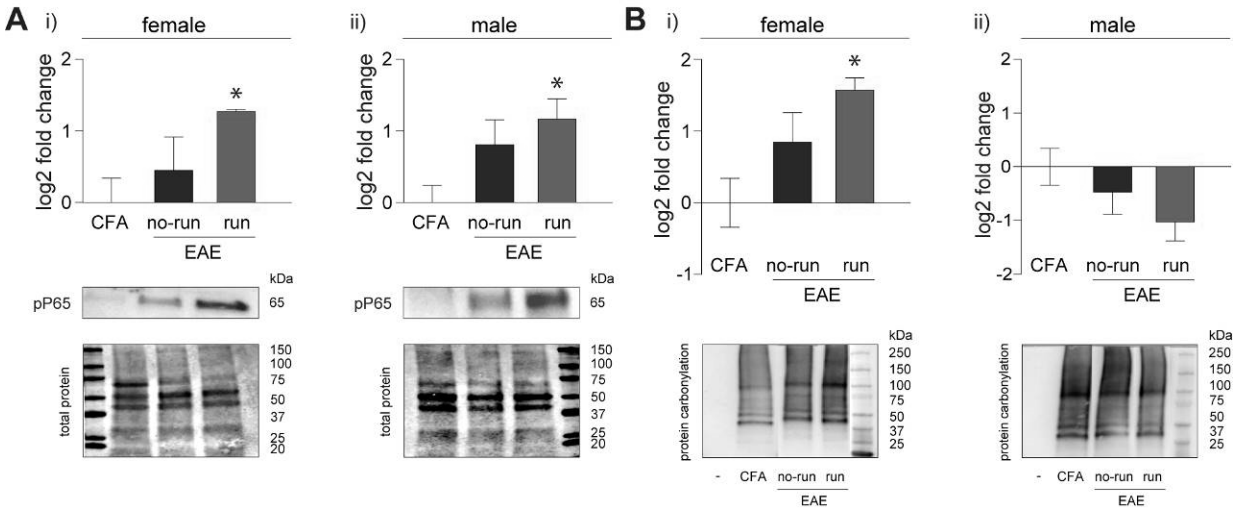

Figure 4.4. *Voluntary wheel running did not alleviate the inflammatory spinal cord environment in EAE.* pP65, a marker of activated macrophages, was used as a measure of spinal cord inflammation. Running was found to increase levels of activated macrophages in both female and male mice (Ai & Aii). Oxidative stress in the spinal cord was assessed by measuring protein carbonylation (B). Female EAE mice showed increased oxidative stress at disease onset, especially in female mice who ran (Bi). No significant differences were found between the male experimental groups (Bii). Bars indicate mean ± standard error of mean (SEM). *$p < 0.05$, one way with tukey's multiple comparisons.

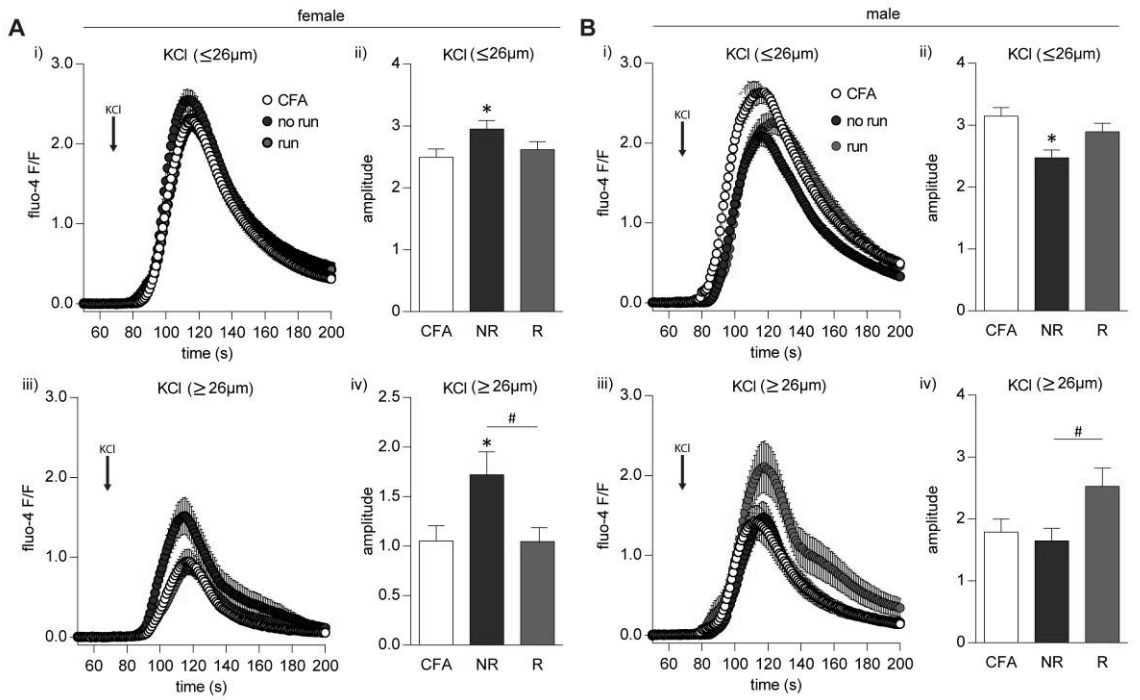

Figure 4.5. *Voluntary wheel running increases male DRG hyperexcitability.* Dorsal root ganglia neuronal cultures were stimulated with KCl to measures potential changes in neuronal excitability associated with running in male and female mice using calcium imaging. Representative traces of response from large (Ai, Bi) and small (Aiii, Biii) diameter neurons are shown. Analysis of amplitude found that non-running EAE female mice had increased activity, as measured by increased change in florescence (Aii,vi). This was especially seen in large diameter neurons (Aiv). Additionally, it was found that the increased in amplitude of large diameter neurons with EAE, as revered by having access to a running wheel (Aiv). Changes were also seen in male small (Bi) and large (Biii) diameter neurons who ran and didn't run. A slight decrease was seen in the amplitude (Bii) in small diameter neurons in EAE mice who did not run. No change was seen in small diameter neurons with running (Bii). Running in EAE male mice also dramatically increased activity of large diameter neurons as measured by amplitude (Biv), which likely is contributing to the nociceptive behaviour seen in male EAE mice. Bars indicate mean ± standard error of mean (SEM). *, # $p < 0.05$, one way ANOVAs with tukey's multiple comparisons.

Figure 6

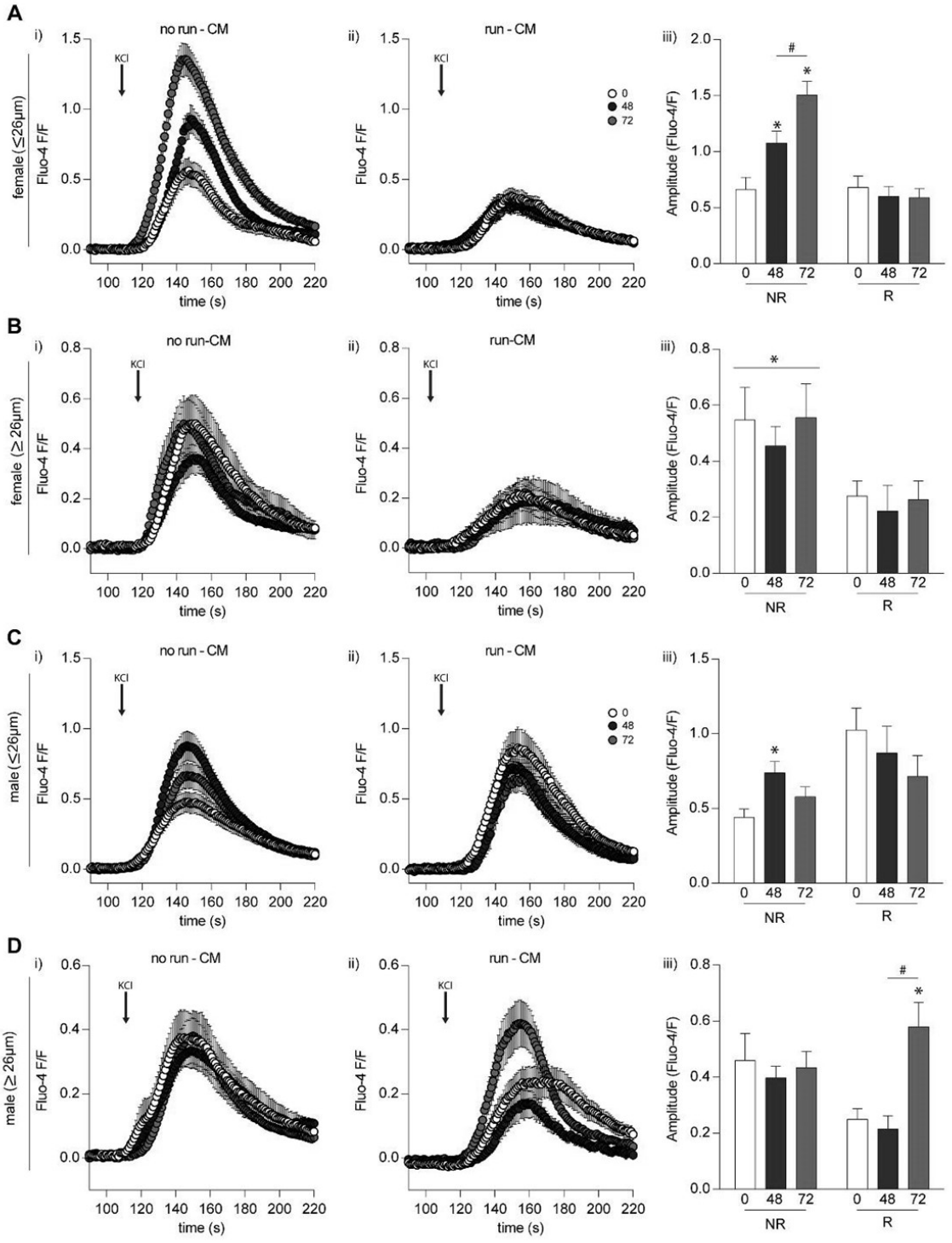
A
i)
no run - CM
KCl
female (≤26µm)
Fluo-4 F/F
time (s)
ii)
run - CM
0
48
72
iii)
Amplitude (Fluo-4/F)
NR
R
B
no run-CM
run-CM
female (≥ 26µm)
C
male(≤26µm)
D
male (≥ 26µm)

Figure 4.6. *Effect of CM on DRG hyperexcitability on naïve mice.* Media from the splenocyte cultures of non-running and running EAE mice of both sexes was used to stimulate DRG cultures from naïve mice. Representative traces from the calcium imaging are seen in Ai, Aii, Bi, Bii, Ci, Cii, Di, and Dii. Naïve female DRG neurons incubated with the 48h and 72h splenocyte media from non-running EAE mice demonstrated an increase in activity in small diameter neurons (Ai, Aiii).. This increase was greatest with the 72h media (Aiii). No differences were seen in small diameter activity with exposure to media from running female EAE mice (Aii, Aiii). Non-running media also increased the activity of large diameter neurons in naïve female mice compared to running media (Bi-Biii). The 48h splenocyte media from non-running mice also increased the activity of small diameter neurons (Ci, Ciii). No effect of running media was seen in small diameter neurons in male naive mice (Cii, Ciii). However, the 72h running media increased the activity of large diameter neurons in male naïve mice (Dii, Diii). No effect of non-running media was seen in the large diameter neurons of male naïve mice (Di, Diii).

4.5 References

Benson C, Paylor JW, Tenorio G, Winship I, Baker G, Kerr BJ (2015) Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE). Exp Neurol 271:279–290 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0014488615300042 [Accessed February 8, 2018].

Bernardes D, Brambilla R, Bracchi-Ricard V, Karmally S, Dellarole A, Carvalho-Tavares J, Bethea JR (2016) Prior regular exercise improves clinical outcome and reduces demyelination and axonal injury in experimental autoimmune encephalomyelitis. J Neurochem 136 Suppl 1:63–73 Available at: http://doi.wiley.com/10.1111/jnc.13354 [Accessed February 8, 2018].

Bernardes D, Oliveira-Lima OC, Silva TV da, Faraco CCF, Leite HR, Juliano MA, Santos DM dos, Bethea JR, Brambilla R, Orian JM, Arantes RME, Carvalho-Tavares J (2013) Differential brain and spinal cord cytokine and BDNF levels in experimental autoimmune encephalomyelitis are modulated by prior and regular exercise. J Neuroimmunol 264:24–34 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572813002373 [Accessed February 8, 2018].

Bernardes D, Oliveira ALR (2017) Comprehensive catwalk gait analysis in a chronic model of multiple sclerosis subjected to treadmill exercise training. BMC Neurol 17:160 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28830377 [Accessed May 15, 2018].

Berta T, Park C-K, Xu Z-Z, Xie R-G, Liu T, Lü N, Liu Y-C, Ji R-R (2014) Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion. J Clin Invest 124:1173–1186 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24531553 [Accessed May 25, 2018].

Berta T, Qadri Y, Tan P-H, Ji R-R (2017) Targeting dorsal root ganglia and primary sensory neurons for the treatment of chronic pain. Expert Opin Ther Targets 21:695–703 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28480765 [Accessed May 28, 2018].

Chen S-X, Liao G-J, Yao P-W, Wang S-K, Li Y-Y, Zeng W, Liu X-G, Zang Y (2018) Calpain-2 Regulates TNF-α expression associated with neuropathic pain following motor nerve injury. Neuroscience 376:142–151 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452218301416 [Accessed May 25, 2018].

Cimpean A, Neacsu P, Mazare A, Schmuki P (2015) Attenuation of the macrophage inflammatory activity by TiO2 nanotubes via inhibition of MAPK and NF-κB pathways. Int J Nanomedicine 10:6455 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26491301 [Accessed June 13, 2018].

Crabtree-Hartman E (2018) Advanced symptom management in multiple sclerosis. Neurol Clin 36:197–218 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0733861917301020?via%3Dihub [Accessed May 15, 2018].

Day Y-J, Liou J-T, Lee C-M, Lin Y-C, Mao C-C, Chou A-H, Liao C-C, Lee H-C (2014) Lack of interleukin-17 leads to a modulated micro-environment and amelioration of mechanical hypersensitivity after peripheral nerve injury in mice. Pain 155:1293–1302 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201407000-00019 [Accessed February 8, 2018].

Dunn SE, Bhat R, Straus DS, Sobel RA, Axtell R, Johnson A, Nguyen K, Mukundan L, Moshkova M, Dugas JC, Chawla A, Steinman L (2010) Peroxisome proliferator-activated receptor delta limits the expansion of pathogenic Th cells during central nervous system autoimmunity. J Exp Med 207:1599–1608 Available at: http://www.jem.org/lookup/doi/10.1084/jem.20091663 [Accessed February 8, 2018].

Dunn SE, Gunde E, Lee H (2015) Sex-based differences in multiple sclerosis (MS): Part II: rising incidence of multiple sclerosis in women and the vulnerability of men to progression of this disease. In, pp 57–86. Springer, Cham. Available at: http://link.springer.com/10.1007/7854_2015_370 [Accessed April 13, 2018].

Dunn SE, Ousman SS, Sobel RA, Zuniga L, Baranzini SE, Youssef S, Crowell A, Loh J, Oksenberg J, Steinman L (2007) Peroxisome proliferator–activated receptor (PPAR)α expression in T cells mediates gender differences in development of T cell–mediated autoimmunity. J Exp Med 204:321–330 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17261635 [Accessed May 23, 2018].

Einstein O, Fainstein N, Touloumi O, Lagoudaki R, Hanya E, Grigoriadis N, Katz A, Ben-Hur T (2018) Exercise training attenuates experimental autoimmune encephalomyelitis by peripheral immunomodulation rather than direct neuroprotection. Exp Neurol 299:56–64 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0014488617302595?via%3Dihub [Accessed April 11, 2018].

Frezel N, Sohet F, Daneman R, Basbaum AI, Braz JM (2016) Peripheral and central neuronal ATF3 precedes CD4 + T-cell infiltration in EAE. Exp Neurol 283:224–234 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0014488616301844?via%3Dihub [Accessed May 30, 2018].

Giacoppo S, Iori R, Bramanti P, Mazzon E (2017) Topical moringin cream relieves neuropathic pain by suppression of inflammatory pathway and voltage-gated ion channels in murine model of multiple sclerosis. Mol Pain 13:174480691772431 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28741431 [Accessed May 25, 2018].

Greenspan JD, Craft RM, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA, Mogil JS, Murphy AZ, Traub RJ, Consensus Working Group of the Sex, Gender, and Pain SIG of the IASP (2007) Studying sex and gender differences in pain and analgesia: A consensus report. Pain 132:S26–S45 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17964077 [Accessed February 8, 2018].

Han Z, Shen F, He Y, Degos V, Camus M, Maze M, Young WL, Su H (2014) Activation of α-7 nicotinic acetylcholine receptor reduces ischemic stroke injury through reduction of pro-inflammatory macrophages and oxidative stress. Annunziato L, ed. PLoS One 9:e105711 Available at: http://dx.plos.org/10.1371/journal.pone.0105711 [Accessed June 13, 2018].

Kalia L V, OConnor PW (2005) Severity of chronic pain and its relationship to quality of life in multiple sclerosis. Mult Scler J 11:322–327 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15957515 [Accessed May 15, 2018].

Kim CF, Moalem-Taylor G (2011) Interleukin-17 contributes to neuroinflammation and neuropathic pain following peripheral nerve injury in mice. J Pain 12:370–383 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20889388 [Accessed May 25, 2018].

Kim T-W, Sung Y-H (2017) Regular exercise promotes memory function and enhances hippocampal neuroplasticity in experimental autoimmune encephalomyelitis mice. Neuroscience 346:173–181 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0306452217300222?via%3Dihub [Accessed May 15, 2018].

Klaren RE, Motl RW, Woods JA, Miller SD (2014) Effects of exercise in experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis). J Neuroimmunol 274:14–19 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572814001854 [Accessed February 8, 2018].

Klaren RE, Stasula U, Steelman AJ, Hernandez J, Pence BD, Woods JA, Motl RW (2016) Effects of exercise in a relapsing-remitting model of experimental autoimmune encephalomyelitis. J Neurosci Res 94:907–914 Available at: http://doi.wiley.com/10.1002/jnr.23783 [Accessed February 8, 2018].

Krames ES (2015) The dorsal root ganglion in chronic pain and as a target for neuromodulation: A Review. Neuromodulation Technol Neural Interface 18:24–32 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25354206 [Accessed May 28, 2018].

Langjahr M, Schubert A-L, Sommer C, Üçeyler N (2018) Increased pro-inflammatory cytokine gene expression in peripheral blood mononuclear cells of patients with polyneuropathies. J Neurol 265:618–627 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29372388 [Accessed May 25, 2018].

Le Page C, Bourdoulous S, Béraud E, Couraud PO, Rieu M, Ferry A (1996) Effect of physical exercise on adoptive experimental auto-immune encephalomyelitis in rats. Eur J Appl Physiol Occup Physiol 73:130–135 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8861681 [Accessed February 8, 2018].

Le Page C, Ferry A, Rieu M (1994) Effect of muscular exercise on chronic relapsing experimental autoimmune encephalomyelitis. J Appl Physiol 77:2341–2347 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7868453 [Accessed February 8, 2018].

Liu H, Dolkas J, Hoang K, Angert M, Chernov A V., Remacle AG, Shiryaev SA, Strongin AY, Nishihara T, Shubayev VI (2015) The alternatively spliced fibronectin CS1 isoform regulates IL-17A levels and mechanical allodynia after peripheral nerve injury. J Neuroinflammation 12:158 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26337825 [Accessed May 25, 2018].

López-Álvarez VM, Modol L, Navarro X, Cobianchi S (2015) Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury. Pain 156:1812–1825 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26090759 [Accessed May 28, 2018].

Mifflin KA, Frieser E, Benson C, Baker G, Kerr BJ (2017) Voluntary wheel running differentially affects disease outcomes in male and female mice with experimental autoimmune encephalomyelitis. J Neuroimmunol 305.

Murphy KL, Bethea JR, Fischer R (2017) Neuropathic pain in multiple sclerosis—Current therapeutic intervention and future treatment perspectives. Codon Publications. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29261265 [Accessed May 15, 2018].

O'Connor AB, Schwid SR, Herrmann DN, Markman JD, Dworkin RH (2008) Pain associated with multiple sclerosis: Systematic review and proposed classification. Pain 137:96–111 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17928147 [Accessed May 15, 2018].

Orton S-M, Herrera BM, Yee IM, Valdar W, Ramagopalan S V, Sadovnick AD, Ebers GC (2006) Sex ratio of multiple sclerosis in Canada: a longitudinal study. neurology.thelancet.com 5 Available at: https://ac-els-cdn-com.login.ezproxy.library.ualberta.ca/S1474442206705816/1-s2.0-S1474442206705816-main.pdf?_tid=04d6de7d-1996-4d35-8c41-e13b428ac534&acdnat=1526429066_99a18db567784e1ae7d084fa7441823c [Accessed May 15, 2018].

Patel DI, White LJ (2013) Effect of 10-day forced treadmill training on neurotrophic factors in experimental autoimmune encephalomyelitis. Appl Physiol Nutr Metab 38:194–199 Available at: http://www.nrcresearchpress.com/doi/10.1139/apnm-2012-0303 [Accessed February 8, 2018].

Pryor WM, Freeman KG, Larson RD, Edwards GL, White LJ (2015) Chronic exercise confers neuroprotection in experimental autoimmune encephalomyelitis. J Neurosci Res 93:697–706 Available at: http://doi.wiley.com/10.1002/jnr.23528 [Accessed February 8, 2018].

Reynolds ER, Ashbaugh AD, Hockenberry BJ, McGrew CA (2018) Multiple sclerosis and exercise. Curr Sports Med Rep 17:31–35 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29315107 [Accessed May 15, 2018].

Rossi S, Furlan R, De Chiara V, Musella A, Lo Giudice T, Mataluni G, Cavasinni F, Cantarella C, Bernardi G, Muzio L, Martorana A, Martino G, Centonze D (2009) Exercise attenuates the clinical, synaptic and dendritic abnormalities of experimental autoimmune encephalomyelitis. Neurobiol Dis 36:51–59 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0969996109001636 [Accessed February 8, 2018].

Shirani A, Zhao Y, Kingwell E, Rieckmann P, Tremlett H (2012) Temporal trends of disability progression in multiple sclerosis: findings from British Columbia, Canada (1975-2009). Mult Scler 18:442–450 Available at: http://journals.sagepub.com/doi/10.1177/1352458511422097 [Accessed May 15, 2018].

Solaro C, Messmer Uccelli M (2011) Management of pain in multiple sclerosis: a pharmacological approach. Nat Rev Neurol 7:519–527 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21844896 [Accessed May 15, 2018].

Sorge RE et al. (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nat Neurosci 18:1081–1083 Available at: http://www.nature.com/articles/nn.4053 [Accessed February 8, 2018].

Souza PS, Gonçalves ED, Pedroso GS, Farias HR, Junqueira SC, Marcon R, Tuon T, Cola M, Silveira PCL, Santos AR, Calixto JB, Souza CT, de Pinho RA, Dutra RC (2017) Physical exercise attenuates experimental autoimmune encephalomyelitis by inhibiting peripheral immune response and blood-brain barrier disruption. Mol Neurobiol 54:4723–4737 Available at: http://link.springer.com/10.1007/s12035-016-0014-0 [Accessed February 8, 2018].

Sun C, Zhang J, Chen L, Liu T, Xu G, Li C, Yuan W, Xu H, Su Z (2017) IL-17 contributed to the neuropathic pain following peripheral nerve injury by promoting astrocyte proliferation and secretion of proinflammatory cytokines. Mol Med Rep 15:89–96 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27959414 [Accessed May 25, 2018].

Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O (2018) Multiple sclerosis. Lancet 391:1622–1636 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0140673618304811?via%3Dihub [Accessed May 15, 2018].

Tian D, Tian M, Zhang L, Zhao P, Cui Y, Li J (2018) High fat diet sensitizes fibromyalgia-like pain behaviors in mice via tumor necrosis factor alpha Ahmad A, ed. PLoS One 13:e0190861 Available at: http://dx.plos.org/10.1371/journal.pone.0190861 [Accessed May 25, 2018].

Venasse M, Edwards T, Pilutti LA (2018) Exploring wellness interventions in rogressive multiple sclerosis: an evidence-based review. Curr Treat Options Neurol 20:13 Available at: http://link.springer.com/10.1007/s11940-018-0497-2 [Accessed May 15, 2018].

Voskuhl RR, Sawalha AH, Itoh Y (2018) Sex chromosome contributions to sex differences in multiple sclerosis susceptibility and progression. Mult Scler J 24:22–31 Available at: http://journals.sagepub.com/doi/10.1177/1352458517737394 [Accessed May 15, 2018].

Wang Y, Feng C, He H, He J, Wang J, Li X, Wang S, Li W, Hou J, Liu T, Fang D, Xie S (2018) Sensitization of TRPV1 receptors by TNF-α orchestrates the development of vincristine-induced pain. Oncol Lett 15:5013–5019 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29552137 [Accessed May 25, 2018].

Wen C, Xu M, Mo C, Cheng Z, Guo Q, Zhu X (2018) JMJD6 exerts function in neuropathic pain by regulating NF-κB following peripheral nerve injury in rats. Int J Mol Med 42:633–642 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29620141 [Accessed May 25, 2018].

Yao C, Weng Z, Zhang J, Feng T, Lin Y, Yao S (2016) Interleukin-17A acts to maintain neuropathic pain through activation of CaMKII/CREB signaling in spinal neurons. Mol Neurobiol 53:3914–3926 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26166359 [Accessed May 25, 2018].

Zhang MA, Ahn JJ, Zhao FL, Selvanantham T, Mallevaey T, Stock N, Correa L, Clark R, Spaner D, Dunn SE (2015) Antagonizing peroxisome proliferator-activated receptor activity selectively enhances Th1 immunity in male mice. J Immunol 195:5189–5202 Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500449 [Accessed February 8, 2018].

Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, Akkermann R, Stanczyk FZ, Prat A, Steinman L, Dunn SE (2012) Peroxisome proliferator-activated receptor (PPAR) and - regulate IFN and IL-17A production by human T cells in a sex-specific way. Proc Natl Acad Sci 109:9505–9510 Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1118458109 [Accessed February 8, 2018].

Conclusions

Chronic pain is a condition that affects millions world-wide and one that is experienced differently by each person affected. Given the unique nature of each chronic pain condition, an effective treatment for many of types of chronic pain remain elusive. One of the reasons that treatment options remain ineffective may be the lack of study of variables, such as sex, that have a large effect on the development of chronic pain. The work in this thesis explored how sex may influence chronic pain in two different models: the formalin model of inflammatory pain and in MS related pain. Large sex effects were seen in the studies conducted in this thesis, demonstrating how sex can dramatically affect potential treatment options. This was first seen in the formalin model where certain drugs were only found to work in one sex to reduce nociceptive behaviour. A similar result was found with the voluntary wheel running paradigm used in the EAE. Voluntary wheel running only significantly reduced pain behavior in female mice, and sex differences were also seen in how exercise modulated the disease course itself. Interestingly the sex difference was the reverse here, where males benefited from voluntary wheel running over time but females did not. In both formalin and MS, the behavioural sex differences were associated with unique biological mechanisms in the sexes. This further highlights the need to study sex when testing potential therapeutics as two different pathways may need to be targeted to treat males and females.

In Chapter 1 of the thesis I found that despite the fact that male and female mice demonstrated similar nociceptive behaviour in the formalin model, they responded differently to treatment with the monoamine oxidase inhibitor phenelzine, and its metabolite PEH. In this study, both drugs reduced nociceptive behaviour in male mice, while only PEH was effective at significantly reducing nociceptive behavior in female mice. I then demonstrated that this was due to a serotonergic dependent nociceptive pathway in male mice and a GABAergic dependent pathway in female mice. While this study demonstrates that different neurotransmitters should be targeted for tonic inflammatory pain, it is not yet known if this difference applies to other models of pain. Studies using models of peripheral pain, cancer pain, or disease related pain could be used to see if the same sex specific pathways apply. Given that the first line therapies for chronic pain conditions are often antidepressants that alter the availability of 5-HT, NA, and GABA, understanding how they might work differently in the sexes in different pain states could have value when developing new therapeutics. Furthermore, while the drugs used in the present

study were effective at reducing nociceptive behavior, other drugs that can manipulate neurotransmitters would need to be used for translational purposes. This is due to the fact that irreversible MAO-inhibitors are associated with several undesirable side effects and not generally tolerated well by patients (Wimbiscus et al., 2010). Finally, it would be interesting to explore potential sex differences in changes in neurotransmitter levels in other areas of the CNS in this study. This would be especially relevant as pain processing is not limited to the spinal cord, and changes in the brainstem or brain could also be relevant to how nociceptive behavior is modulated in this paradigm.

In Chapters 2-4 of the current thesis, sex differences were explored in a model of MS. In the second chapter of the thesis, I was interested in determining if the benefits of voluntary wheel running on disease course and nociceptive behavior in female mice with EAE previously reported by our laboratory (Benson et al., 2015) could be replicated in male mice with EAE. An initial study was designed to explore the effect of voluntary wheel running on disease course and disease parameters in chronic EAE mice. In this study, I found that male mice had reduced clinical scores over time and that this was associated with improvements in axonal damage and oxidative stress when compared to male mice who did not. Interestingly, a benefit of voluntary wheel running on disease outcomes was not seen in female mice at the chronic stage of disease. Instead, I observed increases in clinical score and oxidative stress with prolonged running in female mice with EAE. This study highlights how different exercise regimes are probably needed not only for male and female patients with MS, but potentially also for different types of MS. It would be interesting to compare different types of exercise interventions (e.g. running vs strength training) between the sexes to see if the benefits are different. Furthermore, given that voluntary wheel running showed benefits at the onset of disease in a previous study from our laboratory, it would be interesting to repeat the study and look at markers of inflammation and oxidative stress at different time points (e.g. pre-onset, onset, peak and chronic). This could provide insight into when the negative long-term effects of running in female mice develop. The current study also only explored the effect of long-term voluntary wheel running on one behavioural outcome: clinical score, but it would be interesting to see if sex affected other behavioral variables known to change with MS such as anxiety, depressive behaviors, and cognitive function. Given the research demonstrating that males with MS have increased

cognitive dysfunction, it is likely that sex differences may also be seen in other behavioral parameters (Bove and Chitnis, 2013).

In Chapter 3 I explored whether or not voluntary wheel running had effects in the brains of mice with EAE who ran. I demonstrated that voluntary wheel running could modulate levels of neuroactive steroids associated with the EAE. Specially, female mice with EAE who ran had significantly increased levels of brain pregnenolone compared to males. In contrast, male mice with EAE were found to have significantly higher levels of brain allopregnanolone, a neuroprotective NAS, compared to female mice with EAE regardless of exercise. While this study demonstrates that voluntary wheel running can exert changes in the brain in a sex specific way, it only begins to address how these changes may affect behaviour. Additional studies would be needed to explore a larger range of NASs/NAS metabolites and their effects in more detail. In addition, it would be interesting to pharmacologically manipulate the levels of NASs in the brain to see if the effects of running could be altered. Also, the current analysis used the entire cerebrum for NASs analysis; conducting a similar analysis on more specific areas of the brain, such as the motor cortex, sensory cortex, thalamus, or hippocampus could answer whether changes in NASs are affected by running in a regionally specific manner. Overall, much more work is needed to better understand the effect of exercise in the brain and on NAS levels in EAE and MS.

In the final chapter, Chapter 4, the effect of sex and voluntary wheel running on nociceptive behavior associated with EAE was explored. In this chapter I replicated the finding that voluntary wheel running reduced nociceptive behavior in female mice at the onset of EAE (Benson et al., 2015). This effect was not observed in male mice with the disease who ran. In this study I found that these differences in behavior were associated with the activation of different immune factors in male and female mice who had access to a running wheel. Overall, I demonstrated that male mice who ran had a more pro-inflammatory profile with potentiated levels of TNFα and IL-17 and increased DRG excitability. Female EAE mice who ran however had a reduced pro-inflammatory profile that was associated with a reduction in DRG excitability.

To determine whether the altered DRG excitability in male and female mice with EAE was due to sensitization from circulating cytokines or innate changes in the DRG neurons, I performed a series of experiments using conditioned media from EAE splenocyte cultures. Splenocyte media from EAE mice of both sexes who ran or did not run was collected at the 48-

hour and 72-hour time points and applied to sex-matched DRG cultures from naïve mice. Two timepoints were used as levels of IL-17 are best measured at 48 hours and TNFα at 72 hours. Calcium imaging was then used to assess changes in DRG excitability after application of media. Interestingly, I found that the greatest effects of the CM experiments in both sexes were seen with the 72h CM, suggesting that it is likely TNFα is driving the changes in sensory neuron hyperexcitability. Importantly, I show that in the wheel running condition, it is the female mice who exhibit a striking reduction in the levels of TNFα but significant increases were observed in the males. Taken together, these results indicate that TNFα is a critical cytokine responsible for the nociceptive behaviour observed in the present study. However further studies would be needed to fully validate this hypothesis. One approach to further elucidate the role of TNFα in these processes would be to sequester circulating TNFα using function blocking antibodies in the DRG cultures. Ideally, sequestration of TNFα using this method would reduce the development of DRG hyperexcitability. Anti-TNFα therapies have been effective in the treatment of pain in diseases such as rheumatoid arthritis, suggesting that they may be effect in the pain associated with MS (Mikuls and Weaver, 2003). Unfortunately the use of these therapies in rheumatoid arthritis has recently been associated with many adverse side effects, such as the development of malignant tumors (Mikuls and Weaver, 2003; Berghen et al., 2015). Adverse effects of anti-TNFα have also been reported in MS, where antagonism of TNFα was associated with worsening of disease symptoms in patients, leading to a failed clinical trial (Anon, 1999). While it appears as though direct sequestration of TNFα might not be the best approach for translation, there are promising new drugs that block the actions of TNFα through alternative mechanisms. One such drug is XPro1595, which is a dominant negative protein of TNF that prevents the actions of soluble TNFα without interfering with TNFα receptor function, giving better side effect profiles (Steed et al., 2003; Brambilla et al., 2011). In fact, Xpro1595 has been shown to have beneficial effects on EAE outcomes, meaning that it may be beneficial in pain in MS as well (Brambilla et al., 2011; Murphy et al., 2017)

It would also be interesting to explore changes in pain processing areas in the brain (e.g. thalamus, sensory cortex, periaqueductal gray, rostral ventromedial medulla) as it has been shown that exercise can modulate descending inhibitory pain pathways in the brain (Sluka et al., 2013; Lima et al., 2017). This might be especially relevant in male mice given the observation that increases were observed in the DRG only in the wheel running condition. I found no

significant changes in the excitability of DRG sensory neurons in non-running male mice with EAE despite both male and females exhibiting a similar nociceptive behavioural profile. This suggests that other areas of pain processing, such as the spinal cord or brain, are likely more involved in male EAE pain processing compared to females with EAE. Alternatively, differences in anti-inflammatory cytokines such as IL-10 could be explored in both splenocyte cultures and in the spinal cord to see if this could account for some of the differences observed.

The overall goal of this thesis was to demonstrate the value of including sex as a variable in research, and chronic pain research specifically. I was able to accomplish this by demonstrating significant sex differences in two different models of chronic pain and two different potential treatments. Overall, the inclusion of variables such as sex in pre-clinical studies will ultimately lead to better translation of research and treatment for patients.

Bibliography

ADCC (2005) Progress in Autoimmune Diseases Research. Available at: https://www.niaid.nih.gov/sites/default/files/adccfinal.pdf [Accessed June 7, 2018].

Ahboucha S, Butterworth RF, Pomier-layrargues G, Vincent C, Hassoun Z, Baker GB (2008) Neuroactive steroids and fatigue severity in patients with primary biliary cirrhosis and hepatitis C. Neurogastroenterol Motil 20:671–679 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18282171 [Accessed February 22, 2018].

Aicher SA, Silverman MB, Winkler CW, Bebo BF (2004a) Hyperalgesia in an animal model of multiple sclerosis. Pain 110:560–570 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15288396 [Accessed February 8, 2018].

Aicher SA, Silverman MB, Winkler CW, Bebo BF (2004b) Hyperalgesia in an animal model of multiple sclerosis. Pain 110:560–570 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15288396 [Accessed June 8, 2018].

Alberti T, Barbosa W, Vieira J, Raposo N, Dutra R (2017) (−)-β-Caryophyllene, a CB2 receptor-selective phytocannabinoid, suppresses motor paralysis and neuroinflammation in a murine model of multiple sclerosis. Int J Mol Sci 18:691 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28368293 [Accessed June 13, 2018].

Aletaha D et al. (2010) 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 62:2569–2581 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20872595 [Accessed June 8, 2018].

Aloisi AM, Ceccarelli I, Fiorenzani P, De Padova AM, Massafra C (2004) Testosterone affects formalin-induced responses differently in male and female rats. Neurosci Lett 361:262–264 Available at: https://www.sciencedirect.com/science/article/pii/S0304394003014058?via%3Dihub [Accessed February 8, 2018].

Alonso A, Hernán MA (2008) Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 71:129–135 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18606967 [Accessed June 8, 2018].

Aman MM, Jason Yong R, Kaye AD, Urman RD (2018) Evidence-Based Non-Pharmacological Therapies for Fibromyalgia. Curr Pain Headache Rep 22:33 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29619620 [Accessed June 7, 2018].

Andurkar S V., Gendler L, Gulati A (2012) Tramadol antinociception is potentiated by clonidine through α2-adrenergic and I2-imidazoline but not by endothelin ETA receptors in mice. Eur J Pharmacol 683:109–115 Available at: https://www.sciencedirect.com/science/article/pii/S0014299912002567?via%3Dihub [Accessed February 8, 2018].

Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S (2009) Type 17 T helper cells—origins, features and possible roles in rheumatic disease. Nat Rev Rheumatol 5:325–331 Available at: http://www.nature.com/articles/nrrheum.2009.80 [Accessed February 8, 2018].

Anon (1999) TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. Neurology 53:457–465 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10449104 [Accessed June 8, 2018].

Araki E, Nishikawa T (2010) Oxidative stress: A cause and therapeutic target of diabetic complications. J Diabetes Investig 1:90–96 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24843413 [Accessed June 13, 2018].

Arechiga AF, Habib T, He Y, Zhang X, Zhang Z-Y, Funk A, Buckner JH (2009) Cutting edge: the PTPN22 allelic variant associated with autoimmunity impairs B cell signaling. J Immunol 182:3343–3347 Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.0713370 [Accessed June 8, 2018].

Aslani S, Mahmoudi M, Karami J, Jamshidi AR, Malekshahi Z, Nicknam MH (2016) Epigenetic alterations underlying autoimmune diseases. Autoimmunity 49:69–83 Available at: http://www.tandfonline.com/doi/full/10.3109/08916934.2015.1134511 [Accessed February 8, 2018].

Attal N, Cruccu G, Baron R, Haanpää M, Hansson P, Jensen TS, Nurmikko T, European Federation of Neurological Societies (2010) EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. Eur J Neurol 17:1113-e88 Available at: http://doi.wiley.com/10.1111/j.1468-1331.2010.02999.x [Accessed February 8, 2018].

Austin PJ, Moalem-Taylor G (2010) The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. J Neuroimmunol 229:26–50 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20870295 [Accessed June 7, 2018].

Bean DJ, Johnson MH, Kydd RR (2014) The Outcome of Complex Regional Pain Syndrome Type 1: A Systematic Review. J Pain 15:677–690 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1526590014005653 [Accessed February 8, 2018].

Beggs S, Trang T, Salter MW (2012) P2X4R+ microglia drive neuropathic pain. Nat Neurosci 15:1068–1073 Available at: http://www.nature.com/articles/nn.3155 [Accessed February 8, 2018].

Begolka WS, Vanderlugt CL, Rahbe SM, Miller SD (1998) Differential expression of inflammatory cytokines parallels progression of central nervous system pathology in two clinically distinct models of multiple sclerosis. J Immunol 161:4437–4446 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9780223 [Accessed June 7, 2018].

Begum F, Zhu W, Cortes C, MacNeil B, Namaka M (2013) Elevation of tumor necrosis factor

alpha in dorsal root ganglia and spinal cord is associated with neuroimmune modulation of pain in an animal model of multiple sclerosis. J Neuroimmune Pharmacol 8:677–690 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23483352 [Accessed June 8, 2018].

Benedict RHB, Wahlig E, Bakshi R, Fishman I, Munschauer F, Zivadinov R, Weinstock-Guttman B (2005) Predicting quality of life in multiple sclerosis: accounting for physical disability, fatigue, cognition, mood disorder, personality, and behavior change. J Neurol Sci 231:29–34 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15792817 [Accessed February 8, 2018].

Benson C, Paylor JW, Tenorio G, Winship I, Baker G, Kerr BJ (2015) Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE). Exp Neurol 271:279–290 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0014488615300042 [Accessed February 8, 2018].

Berard JL, Wolak K, Fournier S, David S (2010) Characterization of relapsing-remitting and chronic forms of experimental autoimmune encephalomyelitis in C57BL/6 mice. Glia 58:434–445 Available at: http://doi.wiley.com/10.1002/glia.20935 [Accessed February 8, 2018].

Berghen N, Teuwen L-A, Westhovens R, Verschueren P (2015) Malignancies and anti-TNF therapy in rheumatoid arthritis: a single-center observational cohort study. Clin Rheumatol 34:1687–1695 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26219489 [Accessed June 22, 2018].

Berkley KJ (1997) Sex differences in pain. Behav Brain Sci 20:371-80-513 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10097000 [Accessed February 8, 2018].

Berkley KJ, Zalcman SS, Simon VR (2006) Sex and gender differences in pain and inflammation: a rapidly maturing field. Am J Physiol Integr Comp Physiol 291:R241–R244 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16675636 [Accessed February 8, 2018].

Bernardes D, Brambilla R, Bracchi-Ricard V, Karmally S, Dellarole A, Carvalho-Tavares J, Bethea JR (2016) Prior regular exercise improves clinical outcome and reduces demyelination and axonal injury in experimental autoimmune encephalomyelitis. J Neurochem 136 Suppl 1:63–73 Available at: http://doi.wiley.com/10.1111/jnc.13354 [Accessed February 8, 2018].

Bernardes D, Oliveira-Lima OC, Silva TV da, Faraco CCF, Leite HR, Juliano MA, Santos DM dos, Bethea JR, Brambilla R, Orian JM, Arantes RME, Carvalho-Tavares J (2013) Differential brain and spinal cord cytokine and BDNF levels in experimental autoimmune encephalomyelitis are modulated by prior and regular exercise. J Neuroimmunol 264:24–34 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572813002373 [Accessed February 8, 2018].

Bernardes D, Oliveira ALR (2017) Comprehensive catwalk gait analysis in a chronic model of multiple sclerosis subjected to treadmill exercise training. BMC Neurol 17:160 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28830377 [Accessed May 15, 2018].

Berta T, Park C-K, Xu Z-Z, Xie R-G, Liu T, Lü N, Liu Y-C, Ji R-R (2014) Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion. J Clin Invest 124:1173–1186 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24531553 [Accessed May 25, 2018].

Berta T, Qadri Y, Tan P-H, Ji R-R (2017) Targeting dorsal root ganglia and primary sensory neurons for the treatment of chronic pain. Expert Opin Ther Targets 21:695–703 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28480765 [Accessed May 28, 2018].

Besson M, Matthey A, Daali Y, Poncet A, Vuillemier P, Curatolo M, Zeilhofer HU, Desmeules J (2015) GABAergic modulation in central sensitization in humans. Pain 156:397–404 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201503000-00009 [Accessed February 8, 2018].

Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Peri F, Costa B (2008) Glial TLR4 receptor as new target to treat neuropathic pain: Efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. Glia 56:1312–1319 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18615568 [Accessed June 4, 2018].

Bhalla S, Andurkar S V., Gulati A (2013) Involvement of α_2 -adrenoceptors, imidazoline, and endothelin-A receptors in the effect of agmatine on morphine and oxycodone-induced hypothermia in mice. Fundam Clin Pharmacol 27:498–509 Available at: http://doi.wiley.com/10.1111/j.1472-8206.2012.01046.x [Accessed February 8, 2018].

Bishop A, Hobbs KG, Eguchi A, Jeffrey S, Smallwood L, Pennie C, Anderson J, Estévez AG (2009) Differential sensitivity of oligodendrocytes and motor neurons to reactive nitrogen species: implications for multiple sclerosis. J Neurochem 109:93–104 Available at: http://doi.wiley.com/10.1111/j.1471-4159.2009.05891.x [Accessed February 8, 2018].

Bockbrader HN, Wesche D, Miller R, Chapel S, Janiczek N, Burger P (2010) A comparison of the pharmacokinetics and pharmacodynamics of pregabalin and gabapentin. Clin Pharmacokinet 49:661–669 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20818832 [Accessed June 7, 2018].

Boerboom D, Kumar V, Boyer A, Wang Y, Lambrot R, Zhou X, Rico C, Boehm U, Paquet M, Céleste C, Kimmins S, Bernard DJ (2015) β-catenin stabilization in gonadotropes impairs FSH synthesis in male mice in vivo. Endocrinology 156:323–333 Available at: https://academic.oup.com/endo/article-lookup/doi/10.1210/en.2014-1296 [Accessed February 8, 2018].

Bohren Y, Tessier L-H, Megat S, Petitjean H, Hugel S, Daniel D, Kremer M, Fournel S, Hein L, Schlichter R, Freund-Mercier M-J, Yalcin I, Barrot M (2013) Antidepressants suppress neuropathic pain by a peripheral β2-adrenoceptor mediated anti-TNFα mechanism. Neurobiol Dis 60:39–50 Available at: https://www.sciencedirect.com/science/article/pii/S0969996113002337?via%3Dihub [Accessed February 8, 2018].

Bordet T, Pruss RM (2009) Targeting neuroprotection as an alternative approach to preventing

and treating neuropathic pain. Neurotherapeutics 6:648–662 Available at: http://link.springer.com/10.1016/j.nurt.2009.07.001 [Accessed June 13, 2018].

Boulton AJM, Vinik AI, Arezzo JC, Bril V, Feldman EL, Freeman R, Malik RA, Maser RE, Sosenko JM, Ziegler D, American Diabetes Association (2005) Diabetic neuropathies: a statement by the American Diabetes Association. Diabetes Care 28:956–962 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15793206 [Accessed June 7, 2018].

Bourquin A-F, Süveges M, Pertin M, Gilliard N, Sardy S, Davison AC, Spahn DR, Decosterd I (2006) Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. Pain 122:14.e1-14 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-200605000-00006 [Accessed June 1, 2018].

Bove R, Chitnis T (2013) Sexual disparities in the incidence and course of MS. Clin Immunol 149:201–210 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1521661613000673 [Accessed February 8, 2018].

Brambilla R, Ashbaugh JJ, Magliozzi R, Dellarole A, Karmally S, Szymkowski DE, Bethea JR (2011) Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination. Brain 134:2736–2754 Available at: https://academic.oup.com/brain/article-lookup/doi/10.1093/brain/awr199 [Accessed June 22, 2018].

Bretterklieber A, Painsi C, Avian A, Wutte N, Aberer E (2014) Impaired quality of life in patients with systemic sclerosis compared to the general population and chronic dermatoses. BMC Res Notes 7:594 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25183055 [Accessed June 8, 2018].

Brochet B, Deloire MSA, Ouallet J-C, Salort E, Bonnet M, Jové J, Petry KG (2009) Pain and quality of life in the early stages after multiple sclerosis diagnosis: a 2-year longitudinal study. Clin J Pain 25:211–217 Available at: https://insights.ovid.com/crossref?an=00002508-200903000-00006 [Accessed February 8, 2018].

Butkevich IP, Mikhailenko VA, Vershinina EA, Otellin VA, Aloisi AM (2011) Buspirone before prenatal stress protects against adverse effects of stress on emotional and inflammatory pain-related behaviors in infant rats: Age and sex differences. Brain Res 1419:76–84 Available at: https://www.sciencedirect.com/science/article/pii/S0006899311016192?via%3Dihub [Accessed February 8, 2018].

Calabrese V, Bella R, Testa D, Spadaro F, Scrofani A, Rizza V, Pennisi G (1998) Increased cerebrospinal fluid and plasma levels of ultraweak chemiluminescence are associated with changes in the thiol pool and lipid-soluble fluorescence in multiple sclerosis: the pathogenic role of oxidative stress. Drugs Exp Clin Res 24:125–131 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9825228 [Accessed June 8, 2018].

Calvo M, Dawes JM, Bennett DL (2012) The role of the immune system in the generation of

neuropathic pain. Lancet Neurol 11:629–642 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1474442212701345 [Accessed February 8, 2018].

Campbell GR, Ziabreva I, Reeve AK, Krishnan KJ, Reynolds R, Howell O, Lassmann H, Turnbull DM, Mahad DJ (2011) Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. Ann Neurol 69:481–492 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21446022 [Accessed June 13, 2018].

Cardoso CC de D, Giordani AJ, Wolosker AMB, Souhami L, Manso PG, Dias RS, Segreto HRC, Segreto RA (2012) Protracted hypofractionated radiotherapy for Graves' ophthalmopathy: a pilot study of clinical and radiologic response. Int J Radiat Oncol Biol Phys 82:1285–1291 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0360301610033237 [Accessed June 8, 2018].

Caruso D, D'Intino G, Giatti S, Maschi O, Pesaresi M, Calabrese D, Garcia-Segura L-M, Calza L, Melcangi RC (2010) Sex-dimorphic changes in neuroactive steroid levels after chronic experimental autoimmune encephalomyelitis. J Neurochem 114:921–932 Available at: http://doi.wiley.com/10.1111/j.1471-4159.2010.06825.x [Accessed March 22, 2018].

Caruso D, Melis M, Fenu G, Giatti S, Romano S, Grimoldi M, Crippa D, Marrosu MG, Cavaletti G, Melcangi RC (2014) Neuroactive steroid levels in plasma and cerebrospinal fluid of male multiple sclerosis patients. J Neurochem 130:591–597 Available at: http://doi.wiley.com/10.1111/jnc.12745 [Accessed February 27, 2018].

Cervantes-Durán C, Rocha-González HI, Granados-Soto V (2013) Peripheral and spinal 5-HT receptors participate in the pronociceptive and antinociceptive effects of fluoxetine in rats. Neuroscience 252:396–409 Available at: https://www.sciencedirect.com/science/article/pii/S0306452213007100?via%3Dihub [Accessed February 8, 2018].

Chai J, Herrmann DN, Stanton M, Barbano RL, Logigian EL (2005) Painful small-fiber neuropathy in Sjogren syndrome. Neurology 65:925–927 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16186536 [Accessed June 8, 2018].

Chakrabarti S, Liu N-J, Gintzler AR (2010) Formation of mu-/kappa-opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. Proc Natl Acad Sci U S A 107:20115–20119 Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1009923107 [Accessed June 4, 2018].

Challa SR (2015) Surgical animal models of neuropathic pain: Pros and Cons. Int J Neurosci 125:170–174 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24831263 [Accessed June 1, 2018].

Chang P-C, Pollema-Mays SL, Centeno MV, Procissi D, Contini M, Baria AT, Martina M, Apkarian AV (2014) Role of nucleus accumbens in neuropathic pain: Linked multi-scale evidence in the rat transitioning to neuropathic pain. Pain 155:1128–1139 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24607959 [Accessed February 8, 2018].

Chen H, Perez JN, Constantopoulos E, McKee L, Regan J, Hoyer PB, Brooks HL, Konhilas J (2014) A method to study the impact of chemically-induced ovarian failure on exercise capacity and cardiac adaptation in mice. J Vis Exp Available at: http://www.jove.com/video/51083/a-method-to-study-impact-chemically-induced-ovarian-failure-on [Accessed February 8, 2018].

Chen S-X, Liao G-J, Yao P-W, Wang S-K, Li Y-Y, Zeng W, Liu X-G, Zang Y (2018) Calpain-2 Regulates TNF-α expression associated with neuropathic pain following motor nerve injury. Neuroscience 376:142–151 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452218301416 [Accessed May 25, 2018].

Cheung AC, Lapointe-Shaw L, Kowgier M, Meza-Cardona J, Hirschfield GM, Janssen HLA, Feld JJ (2016) Combined ursodeoxycholic acid (UDCA) and fenofibrate in primary biliary cholangitis patients with incomplete UDCA response may improve outcomes. Aliment Pharmacol Ther 43:283–293 Available at: http://doi.wiley.com/10.1111/apt.13465 [Accessed June 8, 2018].

Ching KH, Burbelo PD, Gonzalez-Begne M, Roberts MEP, Coca A, Sanz I, Iadarola MJ (2011) Salivary anti-Ro60 and anti-Ro52 antibody profiles to diagnose Sjögren's syndrome. J Dent Res 90:445–449 Available at: http://journals.sagepub.com/doi/10.1177/0022034510390811 [Accessed February 8, 2018].

Christianson CA, Corr M, Firestein GS, Mobargha A, Yaksh TL, Svensson CI (2010) Characterization of the acute and persistent pain state present in K/BxN serum transfer arthritis. Pain 151:394–403 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201011000-00023 [Accessed February 8, 2018].

Chu F, Shi M, Zheng C, Shen D, Zhu J, Zheng X, Cui L (2018) The roles of macrophages and microglia in multiple sclerosis and experimental autoimmune encephalomyelitis. J Neuroimmunol 318:1–7 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29606295 [Accessed June 13, 2018].

Cicero TJ, Nock B, O'Connor L, Meyer ER (2002) Role of steroids in sex differences in morphine-induced analgesia: activational and organizational effects. J Pharmacol Exp Ther 300:695–701 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11805235 [Accessed February 9, 2018].

Cimpean A, Neacsu P, Mazare A, Schmuki P (2015) Attenuation of the macrophage inflammatory activity by TiO2 nanotubes via inhibition of MAPK and NF-κB pathways. Int J Nanomedicine 10:6455 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26491301 [Accessed June 13, 2018].

Cines DB, Bussel JB, Liebman HA, Luning Prak ET (2009) The ITP syndrome: pathogenic and clinical diversity. Blood 113:6511–6521 Available at: http://www.bloodjournal.org/cgi/doi/10.1182/blood-2009-01-129155 [Accessed June 8, 2018].

Clark AK, Yip PK, Malcangio M (2009) The liberation of fractalkine in the dorsal horn requires

microglial cathepsin S. J Neurosci 29:6945–6954 Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.0828-09.2009 [Accessed February 8, 2018].

Cobacho N, de la Calle JL, Paíno CL (2014) Dopaminergic modulation of neuropathic pain: Analgesia in rats by a D2-type receptor agonist. Brain Res Bull 106:62–71 Available at: https://www.sciencedirect.com/science/article/pii/S0361923014001002?via%3Dihub [Accessed February 8, 2018].

Coderre TJ, Melzack R (1992) The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 12:3665–3670 Available at: http://www.ncbi.nlm.nih.gov/pubmed/1326610 [Accessed February 8, 2018].

Coderre TJ, Vaccarino AL, Melzack R (1990) Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res 535:155–158 Available at: https://www.sciencedirect.com/science/article/pii/0006899390918355?via%3Dihub [Accessed February 8, 2018].

Coffman RL (2006) Origins of the T(H)1-T(H)2 model: a personal perspective. Nat Immunol 7:539–541 Available at: http://www.nature.com/articles/ni0606-539 [Accessed June 7, 2018].

Compton WM, Volkow ND (2006) Abuse of prescription drugs and the risk of addiction. Drug Alcohol Depend 83:S4–S7 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16563663 [Accessed June 7, 2018].

Conigliaro P, Chimenti MS, Triggianese P, Sunzini F, Novelli L, Perricone C, Perricone R (2016) Autoantibodies in inflammatory arthritis. Autoimmun Rev 15:673–683 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1568997216300519 [Accessed February 8, 2018].

Coull JAM, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438:1017–1021.

Coull JAM, Boudreau D, Bachand K, Prescott SA, Nault F, Sík A, De Koninck P, De Koninck Y (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424:938–942.

Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. Nature 444:894–898 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17167479 [Accessed June 6, 2018].

Crabtree-Hartman E (2018) Advanced symptom management in multiple sclerosis. Neurol Clin 36:197–218 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0733861917301020?via%3Dihub [Accessed May 15, 2018].

Craft RM (n.d.) Sex differences in opioid analgesia: from mouse to man;. Clin J Pain 19:175–186 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12792556 [Accessed February 8, 2018].

Crane JD, Abadi A, Hettinga BP, Ogborn DI, MacNeil LG, Steinberg GR, Tarnopolsky MA (2013) Elevated mitochondrial oxidative stress impairs metabolic adaptations to exercise in skeletal muscle. Moro C, ed. PLoS One 8:e81879 Available at: http://dx.plos.org/10.1371/journal.pone.0081879 [Accessed February 8, 2018].

Crawford C, Lee C, Buckenmaier C, Schoomaker E, Petri R, Jonas W (2014) The Current State of the Science for Active Self-Care Complementary and Integrative Medicine Therapies in the Management of Chronic Pain Symptoms: Lessons Learned, Directions for the Future. Pain Med 15:S104–S113 Available at: https://academic.oup.com/painmedicine/article-lookup/doi/10.1111/pme.12406 [Accessed June 7, 2018].

CSEP (2012) Canadian Physical Activity Guidelines Canadian Physical Activity Guidelines.

Dalakas MC (2015) Future perspectives in target-specific immunotherapies of myasthenia gravis. Ther Adv Neurol Disord 8:316–327 Available at: http://journals.sagepub.com/doi/10.1177/1756285615605700 [Accessed June 8, 2018].

Danieli MG, Gelardi C, Guerra F, Cardinaletti P, Pedini V, Gabrielli A (2016) Cardiac involvement in polymyositis and dermatomyositis. Autoimmun Rev 15:462–465 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26826433 [Accessed June 8, 2018].

Daugherty DJ, Selvaraj V, Chechneva O V., Liu X-B, Pleasure DE, Deng W (2013) A TSPO ligand is protective in a mouse model of multiple sclerosis. EMBO Mol Med 5:891–903 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23681668 [Accessed April 17, 2018].

Davitashvili D, Beridze M, Shakarishvili R, Kiziria M, Sanikidze T (2012) The role of endogenous antiradical protective system in multiple sclerosis. Georgian Med News:11–19 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22665726 [Accessed February 8, 2018].

Day Y-J, Liou J-T, Lee C-M, Lin Y-C, Mao C-C, Chou A-H, Liao C-C, Lee H-C (2014) Lack of interleukin-17 leads to a modulated micro-environment and amelioration of mechanical hypersensitivity after peripheral nerve injury in mice. Pain 155:1293–1302 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201407000-00019 [Accessed February 8, 2018].

De Virgilio A, Greco A, Magliulo G, Gallo A, Ruoppolo G, Conte M, Martellucci S, de Vincentiis M (2016) Polyarteritis nodosa: A contemporary overview. Autoimmun Rev 15:564–570 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26884100 [Accessed June 8, 2018].

Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS, Maixner W (2005) Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum Mol Genet 14:135–143 Available at: http://academic.oup.com/hmg/article/14/1/135/2355796/Genetic-basis-for-individual-

variations-in-pain [Accessed June 6, 2018].

DiMauro S, Schon EA, Carelli V, Hirano M (2013) The clinical maze of mitochondrial neurology. Nat Rev Neurol 9:429–444 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23835535 [Accessed June 13, 2018].

Downs CA, Faulkner MS (2015) Toxic stress, inflammation and symptomatology of chronic complications in diabetes. World J Diabetes 6:554 Available at: http://www.wjgnet.com/1948-9358/full/v6/i4/554.htm [Accessed June 7, 2018].

Dubuis E, Thompson V, Leite MI, Blaes F, Maihöfner C, Greensmith D, Vincent A, Shenker N, Kuttikat A, Leuwer M, Goebel A (2014) Longstanding complex regional pain syndrome is associated with activating autoantibodies against alpha-1a adrenoceptors. Pain 155:2408–2417 Available at: http://insights.ovid.com/crossref?an=00006396-201411000-00023 [Accessed February 8, 2018].

Dubuisson D, Dennis SG (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4:161–174 Available at: http://www.ncbi.nlm.nih.gov/pubmed/564014 [Accessed February 8, 2018].

Dunn SE, Bhat R, Straus DS, Sobel RA, Axtell R, Johnson A, Nguyen K, Mukundan L, Moshkova M, Dugas JC, Chawla A, Steinman L (2010) Peroxisome proliferator-activated receptor delta limits the expansion of pathogenic Th cells during central nervous system autoimmunity. J Exp Med 207:1599–1608 Available at: http://www.jem.org/lookup/doi/10.1084/jem.20091663 [Accessed February 8, 2018].

Dunn SE, Gunde E, Lee H (2015) Sex-Based Differences in Multiple Sclerosis (MS): Part II: Rising incidence of multiple sclerosis in women and the vulnerability of men to progression of this disease. In, pp 57–86. Springer, Cham. Available at: http://link.springer.com/10.1007/7854_2015_370 [Accessed April 13, 2018].

Dunn SE, Ousman SS, Sobel RA, Zuniga L, Baranzini SE, Youssef S, Crowell A, Loh J, Oksenberg J, Steinman L (2007) Peroxisome proliferator–activated receptor (PPAR)α expression in T cells mediates gender differences in development of T cell–mediated autoimmunity. J Exp Med 204:321–330 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17261635 [Accessed May 23, 2018].

Dutta R, McDonough J, Yin X, Peterson J, Chang A, Torres T, Gudz T, Macklin WB, Lewis DA, Fox RJ, Rudick R, Mirnics K, Trapp BD (2006) Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. Ann Neurol 59:478–489 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16392116 [Accessed June 13, 2018].

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice ASC, Stacey BR, Treede R-D, Turk DC, Wallace MS (2007) Pharmacologic management of neuropathic pain: Evidence-based recommendations. Pain 132:237–251 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17920770 [Accessed February 8, 2018].

Ebbinghaus M, Gajda M, Boettger MK, Schaible H-G, Bräuer R (2012) The anti-inflammatory

effects of sympathectomy in murine antigen-induced arthritis are associated with a reduction of Th1 and Th17 responses. Ann Rheum Dis 71:253–261 Available at: http://ard.bmj.com/lookup/doi/10.1136/ard.2011.150318 [Accessed February 8, 2018].

Einstein O, Fainstein N, Touloumi O, Lagoudaki R, Hanya E, Grigoriadis N, Katz A, Ben-Hur T (2018) Exercise training attenuates experimental autoimmune encephalomyelitis by peripheral immunomodulation rather than direct neuroprotection. Exp Neurol 299:56–64 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0014488617302595?via%3Dihub [Accessed April 11, 2018].

Ferjan I, Lipnik-Stangelj M (2013) Chronic pain treatment: the influence of tricyclic antidepressants on serotonin release and uptake in mast cells. Mediat Inflamm 2013:340473.

Ferretti C, La Cava A (2016) Adaptive immune regulation in autoimmune diabetes. Autoimmun Rev 15:236–241 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1568997215002396 [Accessed February 8, 2018].

Ferrini F, Trang T, Mattioli T-AM, Laffray S, Del'Guidice T, Lorenzo L-E, Castonguay A, Doyon N, Zhang W, Godin AG, Mohr D, Beggs S, Vandal K, Beaulieu J-M, Cahill CM, Salter MW, De Koninck Y (2013) Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. Nat Neurosci 16:183–192 Available at: http://www.nature.com/articles/nn.3295 [Accessed February 8, 2018].

Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL (2009) Sex, gender, and pain: a review of recent clinical and experimental findings. J Pain 10:447–485 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19411059 [Accessed February 8, 2018].

Finnerup NB et al. (2015) Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol 14:162–173 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25575710 [Accessed June 7, 2018].

Finnerup NB, Sindrup SH, Jensen TS (2010) The evidence for pharmacological treatment of neuropathic pain. Pain 150:573–581 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20705215 [Accessed February 8, 2018].

Fornasari D (2017) Pharmacotherapy for Neuropathic Pain: A Review. Pain Ther 6:25–33 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29178034 [Accessed June 7, 2018].

Frezel N, Sohet F, Daneman R, Basbaum AI, Braz JM (2016) Peripheral and central neuronal ATF3 precedes CD4 + T-cell infiltration in EAE. Exp Neurol 283:224–234 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0014488616301844?via%3Dihub [Accessed May 30, 2018].

Gazerani P, Wang K, Cairns BE, Svensson P, Arendt-Nielsen L (2006) Effects of subcutaneous administration of glutamate on pain, sensitization and vasomotor responses in healthy men

and women. Pain 124:338–348 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16919390 [Accessed February 8, 2018].

Geneen LJ, Moore RA, Clarke C, Martin D, Colvin LA, Smith BH (2017) Physical activity and exercise for chronic pain in adults: an overview of Cochrane Reviews. Cochrane Database Syst Rev 4:CD011279 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28436583 [Accessed June 7, 2018].

Giacoppo S, Iori R, Bramanti P, Mazzon E (2017) Topical moringin cream relieves neuropathic pain by suppression of inflammatory pathway and voltage-gated ion channels in murine model of multiple sclerosis. Mol Pain 13:174480691772431 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28741431 [Accessed May 25, 2018].

Giatti S, Boraso M, Melcangi RC, Viviani B (2012) Neuroactive steroids, their metabolites, and neuroinflammation. J Mol Endocrinol 49:R125-34 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22966132 [Accessed June 12, 2018].

Giatti S, D'Intino G, Maschi O, Pesaresi M, Garcia-Segura L-M, Calza L, Caruso D, Melcangi RC (2010) Acute experimental autoimmune encephalomyelitis induces sex dimorphic changes in neuroactive steroid levels. Neurochem Int 56:118–127 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19772882 [Accessed April 4, 2018].

Gierthmühlen J, Binder A, Baron R (2014) Mechanism-based treatment in complex regional pain syndromes. Nat Rev Neurol 10:518–528 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25134708 [Accessed June 8, 2018].

Gilgun-Sherki Y, Melamed E, Offen D (2004) The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. J Neurol 251:261–268 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15015004 [Accessed February 8, 2018].

Giltiay N V, Chappell CP, Clark EA (2012) B-cell selection and the development of autoantibodies. Arthritis Res Ther 14 Suppl 4:S1 Available at: http://arthritis-research.biomedcentral.com/articles/10.1186/ar3918 [Accessed June 8, 2018].

Gioiosa L, Chen X, Watkins R, Klanfer N, Bryant CD, Evans CJ, Arnold AP (2008a) Sex chromosome complement affects nociception in tests of acute and chronic exposure to morphine in mice. Horm Behav 53:124–130 Available at: https://www.sciencedirect.com/science/article/pii/S0018506X07002127?via%3Dihub [Accessed February 8, 2018].

Gioiosa L, Chen X, Watkins R, Umeda EA, Arnold AP (2008b) Sex chromosome complement affects nociception and analgesia in newborn mice. J Pain 9:962–969 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18635401 [Accessed February 8, 2018].

Girolomoni G, Mrowietz U, Paul C (2012) Psoriasis: rationale for targeting interleukin-17. Br J Dermatol 167:717–724 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22716185 [Accessed June 7, 2018].

Gisondi P, Dalle Vedove C, Girolomoni G (2014) Efficacy and Safety of Secukinumab in

Chronic Plaque Psoriasis and Psoriatic Arthritis Therapy. Dermatol Ther (Heidelb) 4:1–9 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24452484 [Accessed June 7, 2018].

Goebel A (2016) Autoantibody pain. Autoimmun Rev 15:552–557 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1568997216300374 [Accessed June 8, 2018].

Goebel A, Blaes F (2013) Complex regional pain syndrome, prototype of a novel kind of autoimmune disease. Autoimmun Rev 12:682–686 Available at: http://linkinghub.elsevier.com/retrieve/pii/S156899721200273X [Accessed June 8, 2018].

Grace PM, Rolan PE, Hutchinson MR (2011) Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. Brain Behav Immun 25:1322–1332 Available at: http://linkinghub.elsevier.com/retrieve/pii/S088915911100119X [Accessed February 8, 2018].

Grant SL, Shulman Y, Tibbo P, Hampson DR, Baker GB (2006) Determination of d-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection. J Chromatogr B Analyt Technol Biomed Life Sci 844:278–282 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16890503 [Accessed February 8, 2018].

Greco CM, Rudy TE, Manzi S (2003) Adaptation to chronic pain in systemic lupus erythematosus: applicability of the multidimensional pain inventory. Pain Med 4:39–50 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12873277 [Accessed June 8, 2018].

Green PHR, Krishnareddy S, Lebwohl B (2015) Clinical Manifestations of Celiac Disease. Dig Dis 33:137–140 Available at: https://www.karger.com/Article/FullText/370204 [Accessed February 8, 2018].

Greenspan JD, Craft RM, LeResche L, Arendt-Nielsen L, Berkley KJ, Fillingim RB, Gold MS, Holdcroft A, Lautenbacher S, Mayer EA, Mogil JS, Murphy AZ, Traub RJ, Consensus Working Group of the Sex, Gender, and Pain SIG of the IASP (2007) Studying sex and gender differences in pain and analgesia: A consensus report. Pain 132:S26–S45 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17964077 [Accessed February 8, 2018].

Gregory NS, Harris AL, Robinson CR, Dougherty PM, Fuchs PN, Sluka KA (2013) An Overview of Animal Models of Pain: Disease Models and Outcome Measures. J Pain 14:1255–1269 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S1526590013010985?via%3Dihub#bib74 [Accessed June 1, 2018].

Grossman S, Tagliavini LB (2015) Managing Sjogren's Syndrome. Home Healthc now 33:487–492 Available at: http://insights.ovid.com/crossref?an=01845097-201510000-00007 [Accessed June 7, 2018].

Hache G, Guiard BP, Nguyen TH, Quesseveur G, Gardier AM, Peters D, Munro G, Coudoré F (2015) Antinociceptive activity of the new triple reuptake inhibitor NS18283 in a mouse model of chemotherapy-induced neuropathic pain. Eur J Pain 19:322–333 Available at:

http://doi.wiley.com/10.1002/ejp.550 [Accessed February 8, 2018].

Haider L (2015) Inflammation, iron, energy failure, and oxidative stress in the pathogenesis of multiple sclerosis. Oxid Med Cell Longev 2015:725370 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26106458 [Accessed June 13, 2018].

Hains BC, Everhart AW, Fullwood SD, Hulsebosch CE (2002) Changes in serotonin, serotonin transporter expression and serotonin denervation supersensitivity: Involvement in chronic central pain after spinal hemisection in the rat. Exp Neurol 175:347–362 Available at: https://www.sciencedirect.com/science/article/pii/S0014488602978921?via%3Dihub [Accessed February 8, 2018].

Hama A, Takamatsu H (2016) Chemotherapy-induced peripheral neuropathic pain and rodent models. CNS Neurol Disord - Drug Targets 15:7–19 Available at: http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1871-5273&volume=15&issue=1&spage=7 [Accessed June 1, 2018].

Han Z, Shen F, He Y, Degos V, Camus M, Maze M, Young WL, Su H (2014) Activation of α-7 nicotinic acetylcholine receptor reduces ischemic stroke injury through reduction of pro-inflammatory macrophages and oxidative stress. Annunziato L, ed. PLoS One 9:e105711 Available at: http://dx.plos.org/10.1371/journal.pone.0105711 [Accessed June 13, 2018].

Harden RN, Bruehl S, Stanton-Hicks M, Wilson PR (2007) Proposed new diagnostic criteria for complex regional pain syndrome. Pain Med 8:326–331 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17610454 [Accessed June 8, 2018].

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88 Available at: http://www.ncbi.nlm.nih.gov/pubmed/3340425 [Accessed June 1, 2018].

Hathway GJ, Vega-Avelaira D, Moss A, Ingram R, Fitzgerald M (2009) Brief, low frequency stimulation of rat peripheral C-fibres evokes prolonged microglial-induced central sensitization in adults but not in neonates. Pain 144:110–118 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-200907000-00021 [Accessed February 8, 2018].

Hayashi S, Taketa Y, Inoue K, Takahashi M, Matsuo S, Irie K, Watanabe G, Yoshida M (2013) Effects of piperonyl butoxide on the female reproductive tract in rats. J Toxicol Sci 38:891–902 Available at: http://jlc.jst.go.jp/DN/JST.JSTAGE/jts/38.891?lang=en&from=CrossRef&type=abstract [Accessed February 8, 2018].

Hayashida K-I, Bynum T, Vincler M, Eisenach JC (2006) Inhibitory M2 muscarinic receptors are upregulated in both axotomized and intact small diameter dorsal root ganglion cells after peripheral nerve injury. Neuroscience 140:259–268 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452206001874 [Accessed June 8, 2018].

Hinson SR, Roemer SF, Lucchinetti CF, Fryer JP, Kryzer TJ, Chamberlain JL, Howe CL, Pittock SJ, Lennon VA (2008) Aquaporin-4–binding autoantibodies in patients with neuromyelitis

optica impair glutamate transport by down-regulating EAAT2. J Exp Med 205:2473–2481 Available at: http://www.jem.org/lookup/doi/10.1084/jem.20081241 [Accessed February 8, 2018].

Hocking LJ, Smith BH, Jones GT, Reid DM, Strachan DP, Macfarlane GJ (2010) Genetic variation in the beta2-adrenergic receptor but not catecholamine-O-methyltransferase predisposes to chronic pain: Results from the 1958 British Birth Cohort Study. Pain 149:143–151 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201004000-00022 [Accessed February 8, 2018].

Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP, Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF (2006) A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. J Pharmacol Exp Ther 319:1376–1385 Available at: http://jpet.aspetjournals.org/cgi/doi/10.1124/jpet.106.111559 [Accessed June 7, 2018].

Howe CL, Kaptzan T, Magaña SM, Ayers-Ringler JR, LaFrance-Corey RG, Lucchinetti CF (2014) Neuromyelitis optica IgG stimulates an immunological response in rat astrocyte cultures. Glia 62:692–708 Available at: http://doi.wiley.com/10.1002/glia.22635 [Accessed February 8, 2018].

Hueber W et al. (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. Sci Transl Med 2:52ra72-52ra72 Available at: http://stm.sciencemag.org/cgi/doi/10.1126/scitranslmed.3001107 [Accessed February 8, 2018].

Hughes SW, Hickey L, Hulse RP, Lumb BM, Pickering AE (2013a) Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. Pain 154:1680–1690 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201309000-00025 [Accessed February 8, 2018].

Hughes SW, Hickey L, Hulse RP, Lumb BM, Pickering AE (2013b) Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. Pain 154:1680–1690 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23707289 [Accessed February 8, 2018].

Hüllemann P, Keller T, Kabelitz M, Gierthmühlen J, Freynhagen R, Tölle T, Forstenpointner J, Baron R (2018) Clinical manifestation of acute, subacute and chronic low back pain in different age groups. Pain Pract Available at: http://www.ncbi.nlm.nih.gov/pubmed/29710429 [Accessed June 5, 2018].

Iadarola MJ, Brady LS, Draisci G, Dubner R (1988) Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. Pain 35:313–326 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/2906426 [Accessed June 1, 2018].

Inoue A, Koh CS, Yahikozawa H, Yanagisawa N, Yagita H, Ishihara Y, Kim BS (1996) The level of tumor necrosis factor-alpha producing cells in the spinal cord correlates with the degree of Theiler's murine encephalomyelitis virus-induced demyelinating disease. Int Immunol 8:1001–1008 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8757945 [Accessed June 13, 2018].

Institute of Medicine (US) Committee on Advancing Pain Research, Care and E (2011) Relieving Pain in America. Washington, D.C.: National Academies Press. Available at: http://www.nap.edu/catalog/13172 [Accessed April 30, 2018].

Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, Peles E, Buckley C, Lang B, Vincent A (2010) Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain 133:2734–2748 Available at: https://academic.oup.com/brain/article-lookup/doi/10.1093/brain/awq213 [Accessed February 8, 2018].

Jasmin L, Rabkin SD, Granato A, Boudah A, Ohara PT (2003) Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424:316–320.

Jiménez-Rivera C et al. (2015) Incidence and characteristics of autoimmune hepatitis. Pediatrics 136:e1237-48 Available at: http://pediatrics.aappublications.org/cgi/doi/10.1542/peds.2015-0578 [Accessed June 8, 2018].

Johannes CB, Le TK, Zhou X, Johnston JA, Dworkin RH (2010) The Prevalence of chronic pain in United States adults: Results of an internet-based survey. J Pain 11:1230–1239 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S1526590010006012 [Accessed June 5, 2018].

Joseph EK, Chen X, Bogen O, Levine JD (2008) Oxaliplatin acts on IB4-positive nociceptors to induce an oxidative stress-dependent acute painful peripheral neuropathy. J Pain 9:463–472 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1526590008003891 [Accessed June 13, 2018].

Jousse-Joulin S, Morvan J, Devauchelle-Pensec V, Saraux A (2013) Ultrasound assessment of the entheses in primary Sjögren syndrome. Ultrasound Med Biol 39:2485–2487 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24035411 [Accessed June 7, 2018].

Juni A, Cai M, Stankova M, Waxman AR, Arout C, Klein G, Dahan A, Hruby VJ, Mogil JS, Kest B (2010) Sex-specific mediation of opioid-induced hyperalgesia by the melanocortin-1 receptor. Anesthesiology 112:181–188 Available at: http://anesthesiology.pubs.asahq.org/Article.aspx?doi=10.1097/ALN.0b013e3181c53849 [Accessed February 8, 2018].

Kalia L V, OConnor PW (2005) Severity of chronic pain and its relationship to quality of life in multiple sclerosis. Mult Scler J 11:322–327 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/15957515 [Accessed May 15, 2018].

Kang S-Y, Kim C-Y, Roh D-H, Yoon S-Y, Park J-H, Lee H-J, Beitz AJ, Lee J-H (2011) Chemical stimulation of the ST36 acupoint reduces both formalin-induced nociceptive behaviors and spinal astrocyte activation via spinal alpha-2 adrenoceptors. Brain Res Bull 86:412–421 Available at: https://www.sciencedirect.com/science/article/pii/S0361923011002590?via%3Dihub [Accessed February 8, 2018].

Kato K, Sullivan PF, Evengård B, Pedersen NL (2006) Importance of genetic influences on chronic widespread pain. Arthritis Rheum 54:1682–1686 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16646040 [Accessed June 6, 2018].

Khan N, Gordon R, Woodruff TM, Smith MT (2015) Antiallodynic effects of alpha lipoic acid in an optimized RR-EAE mouse model of MS-neuropathic pain are accompanied by attenuation of upregulated BDNF-TrkB-ERK signaling in the dorsal horn of the spinal cord. Pharmacol Res Perspect 3:e00137 Available at: http://doi.wiley.com/10.1002/prp2.137 [Accessed February 8, 2018].

Kim CF, Moalem-Taylor G (2011) Interleukin-17 Contributes to Neuroinflammation and Neuropathic Pain Following Peripheral Nerve Injury in Mice. J Pain 12:370–383 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20889388 [Accessed May 25, 2018].

Kim T-W, Sung Y-H (2017) Regular exercise promotes memory function and enhances hippocampal neuroplasticity in experimental autoimmune encephalomyelitis mice. Neuroscience 346:173–181 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0306452217300222?via%3Dihub [Accessed May 15, 2018].

Kim J-Y V, Tillu D V, Quinn TL, Mejia GL, Shy A, Asiedu MNK, Murad E, Schumann AP, Totsch SK, Sorge RE, Mantyh PW, Dussor G, Price TJ (2015) Spinal dopaminergic projections control the transition to pathological pain plasticity via a D1/D5-mediated mechanism. J Neurosci 35:6307–6317 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25904784 [Accessed February 8, 2018].

Kingwell E, Zhu F, Marrie RA, Fisk JD, Wolfson C, Warren S, Profetto-McGrath J, Svenson LW, Jette N, Bhan V, Yu BN, Elliott L, Tremlett H (2015) High incidence and increasing prevalence of multiple sclerosis in British Columbia, Canada: findings from over two decades (1991–2010). J Neurol 262:2352–2363 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26205633 [Accessed June 8, 2018].

Kishi T, Miyamae T, Hara R, Nakajima S, Imagawa T, Mori M, Yokota S (2013) Clinical analysis of 50 children with juvenile dermatomyositis. Mod Rheumatol 23:311–317 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22526832 [Accessed June 8, 2018].

Klaren RE, Motl RW, Woods JA, Miller SD (2014) Effects of exercise in experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis). J Neuroimmunol 274:14–19 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572814001854 [Accessed February 8, 2018].

Klaren RE, Stasula U, Steelman AJ, Hernandez J, Pence BD, Woods JA, Motl RW (2016) Effects of exercise in a relapsing-remitting model of experimental autoimmune encephalomyelitis. J Neurosci Res 94:907–914 Available at: http://doi.wiley.com/10.1002/jnr.23783 [Accessed February 8, 2018].

Klein CJ, Lennon VA, Aston PA, McKeon A, Pittock SJ (2012) Chronic pain as a manifestation of potassium channel-complex autoimmunity. Neurology 79:1136–1144 Available at: http://www.neurology.org/cgi/doi/10.1212/WNL.0b013e3182698cab [Accessed February 8, 2018].

Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H, Zeilhofer HU (2008) Reversal of pathological pain through specific spinal GABAAreceptor subtypes. Nature 451:330–334.

Knabl J, Zeilhofer UB, Crestani F, Rudolph U, Zeilhofer HU (2009) Genuine antihyperalgesia by systemic diazepam revealed by experiments in GABAAreceptor point-mutated mice. Pain 141:233–238 Available at: http://dx.doi.org/10.1016/j.pain.2008.10.015.

Kohr D, Singh P, Tschernatsch M, Kaps M, Pouokam E, Diener M, Kummer W, Birklein F, Vincent A, Goebel A, Wallukat G, Blaes F (2011) Autoimmunity against the β2 adrenergic receptor and muscarinic-2 receptor in complex regional pain syndrome. Pain 152:2690–2700 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201112000-00008 [Accessed February 8, 2018].

Krames ES (2015) The dorsal root ganglion in chronic pain and as a target for neuromodulation: A review. Neuromodulation Technol Neural Interface 18:24–32 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25354206 [Accessed May 28, 2018].

Kremer M, Salvat E, Muller A, Yalcin I, Barrot M (2016) Antidepressants and gabapentinoids in neuropathic pain: Mechanistic insights. Neuroscience 338:183–206 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27401055 [Accessed June 7, 2018].

Krishnamurthy A et al. (2016) Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. Ann Rheum Dis 75:721–729 Available at: http://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2015-208093 [Accessed February 8, 2018].

Kuba T, Wu H-BK, Nazarian A, Festa ED, Barr GA, Jenab S, Inturrisi CE, Quinones-Jenab V (2006) Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Horm Behav 49:441–449 Available at: https://www.sciencedirect.com/science/article/pii/S0018506X05002187?via%3Dihub [Accessed February 8, 2018].

Kumar A, Kaur H, Singh A (2018) Neuropathic Pain models caused by damage to central or peripheral nervous system. Pharmacol Reports 70:206–216 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S1734114017302190?via%3Dihub

[Accessed June 1, 2018].

Langjahr M, Schubert A-L, Sommer C, Üçeyler N (2018) Increased pro-inflammatory cytokine gene expression in peripheral blood mononuclear cells of patients with polyneuropathies. J Neurol 265:618–627 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29372388 [Accessed May 25, 2018].

Le Page C, Bourdoulous S, Béraud E, Couraud PO, Rieu M, Ferry A (1996) Effect of physical exercise on adoptive experimental auto-immune encephalomyelitis in rats. Eur J Appl Physiol Occup Physiol 73:130–135 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8861681 [Accessed February 8, 2018].

Le Page C, Ferry A, Rieu M (1994) Effect of muscular exercise on chronic relapsing experimental autoimmune encephalomyelitis. J Appl Physiol 77:2341–2347 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7868453 [Accessed February 8, 2018].

Lebwohl M, Swensen AR, Nyirady J, Kim E, Gwaltney CJ, Strober BE (2014) The Psoriasis Symptom Diary: development and content validity of a novel patient-reported outcome instrument. Int J Dermatol 53:714–722 Available at: http://doi.wiley.com/10.1111/j.1365-4632.2012.05798.x [Accessed June 8, 2018].

Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR (2005) IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. J Exp Med 202:473–477 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16087714 [Accessed June 8, 2018].

Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, Braun D, Banerjee S (2012) Anti–Interleukin-17 monoclonal antibody Ixekizumab in chronic plaque psoriasis. N Engl J Med 366:1190–1199 Available at: http://www.nejm.org/doi/abs/10.1056/NEJMoa1109997 [Accessed February 8, 2018].

Li J, Simone DA, Larson AA (1999) Windup leads to characteristics of central sensitization. Pain 79:75–82 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9928779 [Accessed June 1, 2018].

Li J, Wei G-H, Huang H, Lan Y-P, Liu B, Liu H, Zhang W, Zuo Y-X (2013) Nerve injury-related autoimmunity activation leads to chronic inflammation and chronic neuropathic pain. Anesthesiology 118:416–429 Available at: http://insights.ovid.com/crossref?an=00000542-201302000-00027 [Accessed February 8, 2018].

Li W-W, Guo T-Z, Shi X, Czirr E, Stan T, Sahbaie P, Wyss-Coray T, Kingery WS, Clark DJ (2014) Autoimmunity contributes to nociceptive sensitization in a mouse model of complex regional pain syndrome. Pain 155:2377–2389 Available at: http://insights.ovid.com/crossref?an=00006396-201411000-00020 [Accessed February 8, 2018].

Li Y-F, Zhang S-X, Ma X-W, Xue Y-L, Gao C, Li X-Y (2017) Levels of peripheral Th17 cells and serum Th17-related cytokines in patients with multiple sclerosis: A meta-analysis. Mult Scler Relat Disord 18:20–25 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29141810

[Accessed June 13, 2018].

Lima L V., Abner TSS, Sluka KA (2017) Does exercise increase or decrease pain? Central mechanisms underlying these two phenomena. J Physiol 595:4141–4150 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28369946 [Accessed June 7, 2018].

Lin C-Y, Lin M-T, Cheng R-T, Chen S-H (2010) Testosterone depletion by castration may protect mice from heat-induced multiple organ damage and lethality. J Biomed Biotechnol 2010:1–9 Available at: http://www.hindawi.com/journals/bmri/2010/485306/ [Accessed February 8, 2018].

Lin Y-C, Wan L, Jamison RN (2017) Using integrative medicine in pain management. Anesth Analg 125:2081–2093 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29189365 [Accessed June 7, 2018].

Lisi L, Navarra P, Cirocchi R, Sharp A, Stigliano E, Feinstein DL, Dello Russo C (2012) Rapamycin reduces clinical signs and neuropathic pain in a chronic model of experimental autoimmune encephalomyelitis. J Neuroimmunol 243:43–51 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0165572811003705?via%3Dihub [Accessed June 8, 2018].

Liu H, Dolkas J, Hoang K, Angert M, Chernov A V., Remacle AG, Shiryaev SA, Strongin AY, Nishihara T, Shubayev VI (2015) The alternatively spliced fibronectin CS1 isoform regulates IL-17A levels and mechanical allodynia after peripheral nerve injury. J Neuroinflammation 12:158 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26337825 [Accessed May 25, 2018].

Liu XY, Wu SX, Wang YY, Wang W, Zhou L, Li YQ (2005) Changes of 5-HT receptor subtype mRNAs in rat dorsal root ganglion by bee venom-induced inflammatory pain. Neurosci Lett 375:42–46.

Loeser JD, Treede RD (2008) The Kyoto protocol of IASP Basic Pain Terminology. Pain 137:473–477.

López-Álvarez VM, Modol L, Navarro X, Cobianchi S (2015) Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury. Pain 156:1812–1825 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26090759 [Accessed May 28, 2018].

Loyd DR, Murphy AZ (2006) Sex differences in the anatomical and functional organization of the periaqueductal gray-rostral ventromedial medullary pathway in the rat: A potential circuit mediating the sexually dimorphic actions of morphine. J Comp Neurol 496:723–738 Available at: http://doi.wiley.com/10.1002/cne.20962 [Accessed February 8, 2018].

Loyd DR, Murphy AZ (2014) The neuroanatomy of sexual dimorphism in opioid analgesia. Exp Neurol 259:57–63 Available at: https://www.sciencedirect.com/science/article/pii/S0014488614000995?via%3Dihub

[Accessed February 8, 2018].

Lublin F (2005) History of modern multiple sclerosis therapy. J Neurol 252 Suppl 3:iii3-iii9 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16170498 [Accessed February 8, 2018].

Luongo L, Sajic M, Grist J, Clark AK, Maione S, Malcangio M (2008) Spinal changes associated with mechanical hypersensitivity in a model of Guillain–Barré syndrome. Neurosci Lett 437:98–102 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304394008004849 [Accessed February 8, 2018].

Lynch JL, Gallus NJ, Ericson ME, Beitz AJ (2008) Analysis of nociception, sex and peripheral nerve innervation in the TMEV animal model of multiple sclerosis. Pain 136:293–304 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17766043 [Accessed February 8, 2018].

Maestroni GJM (2006) Sympathetic nervous system influence on the innate immune response. Ann N Y Acad Sci 1069:195–207 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16855146 [Accessed June 8, 2018].

Mahad D, Ziabreva I, Lassmann H, Turnbull D (2008) Mitochondrial defects in acute multiple sclerosis lesions. Brain 131:1722–1735 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18515320 [Accessed June 13, 2018].

Maimone D, Gregory S, Arnason BG, Reder AT (1991) Cytokine levels in the cerebrospinal fluid and serum of patients with multiple sclerosis. J Neuroimmunol 32:67–74 Available at: http://www.ncbi.nlm.nih.gov/pubmed/2002092 [Accessed June 13, 2018].

Makris A, Piperopoulos A, Karmaniolou I (2014) Multiple sclerosis: basic knowledge and new insights in perioperative management. J Anesth 28:267–278 Available at: http://link.springer.com/10.1007/s00540-013-1697-2 [Accessed June 8, 2018].

Marrie RA, Fisk JD, Stadnyk KJ, Yu BN, Tremlett H, Wolfson C, Warren S, Bhan V, Sclerosis CT in the E and I of C on M (2013) The Incidence and Prevalence of Multiple Sclerosis in Nova Scotia, Canada. Can J Neurol Sci 40:824–831 Available at: http://www.journals.cambridge.org/abstract_S0317167100015961 [Accessed June 8, 2018].

Marrie RA, Yu N, Blanchard J, Leung S, Elliott L (2010) The rising prevalence and changing age distribution of multiple sclerosis in Manitoba. Neurology 74:465–471 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20071664 [Accessed June 8, 2018].

Martindale J, Bland-Ward PA, Chessell IP (2001) Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. Neurosci Lett 316:33–36 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11720772 [Accessed June 1, 2018].

McCarson KE, Enna SJ (2014) GABA Pharmacology: The Search for Analgesics. Neurochem Res 39:1948–1963 Available at: http://link.springer.com/10.1007/s11064-014-1254-x [Accessed February 8, 2018].

McKenna K, Baker G, Coutts R (1991) N2-Acetylphenelzine : effects on rat brain GABA, alanine and biogenic amines. Naunyn Schmiedebergs Arch Pharmacol 343 Available at: http://link.springer.com/10.1007/BF00169549 [Accessed February 8, 2018].

McLean AC, Valenzuela N, Fai S, Bennett SAL (2012) Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp:e4389 Available at: http://www.jove.com/video/4389/performing-vaginal-lavage-crystal-violet-staining-vaginal-cytological [Accessed February 8, 2018].

McMahon SB, La Russa F, Bennett DLH (2015) Crosstalk between the nociceptive and immune systems in host defence and disease. Nat Rev Neurosci 16:389–402 Available at: http://www.nature.com/articles/nrn3946 [Accessed June 8, 2018].

McManus DJ, Baker GB, Martin IL, Greenshaw AJ, McKenna KF (1992) Effects of the antidepressant/antipanic drug phenelzine on GABA concentrations and GABA-transaminase activity in rat brain. Biochem Pharmacol 43:2486–2489 Available at: http://www.ncbi.nlm.nih.gov/pubmed/1610412 [Accessed February 8, 2018].

Meeus M, Nijs J, Hermans L, Goubert D, Calders P (2013) The role of mitochondrial dysfunctions due to oxidative and nitrosative stress in the chronic pain or chronic fatigue syndromes and fibromyalgia patients: peripheral and central mechanisms as therapeutic targets? Expert Opin Ther Targets 17:1081–1089 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23834645 [Accessed June 13, 2018].

Meffre E, Wardemann H (2008) B-cell tolerance checkpoints in health and autoimmunity. Curr Opin Immunol 20:632–638 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0952791508001775 [Accessed June 8, 2018].

Melanson M, Miao P, Eisenstat D, Gong Y, Gu X, Au K, Zhu W, Begum F, Frost E, Namaka M (2009) Experimental autoimmune encephalomyelitis-induced upregulation of tumor necrosis factor-alpha in the dorsal root ganglia. Mult Scler J 15:1135–1145 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19667008 [Accessed June 8, 2018].

Melcangi RC, Panzica G, Garcia-Segura LM (2011) Neuroactive steroids: focus on human brain. Neuroscience 191:1–5 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21704130 [Accessed April 17, 2018].

Menard L, Saadoun D, Isnardi I, Ng Y-S, Meyers G, Massad C, Price C, Abraham C, Motaghedi R, Buckner JH, Gregersen PK, Meffre E (2011) The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. J Clin Invest 121:3635–3644 Available at: http://www.jci.org/articles/view/45790 [Accessed June 8, 2018].

Meske DS, Xie JY, Oyarzo J, Badghisi H, Ossipov MH, Porreca F (2014) Opioid and noradrenergic contributions of tapentadol in experimental neuropathic pain. Neurosci Lett 562:91–96 Available at: http://dx.doi.org/10.1016/j.neulet.2013.08.017.

Mifflin KA, Frieser E, Benson C, Baker G, Kerr BJ (2017) Voluntary wheel running differentially affects disease outcomes in male and female mice with experimental autoimmune encephalomyelitis. J Neuroimmunol 305, 135-144.

Mifflin KA, Kerr BJ (2014) The transition from acute to chronic pain: understanding how different biological systems interact. Can J Anesth Can d'anesthésie 61:112–122 Available

at: http://link.springer.com/10.1007/s12630-013-0087-4 [Accessed February 8, 2018].

Mifflin K, Benson C, Kerr B, Aricioglu F, Cetin M, Dursun S, Baker G (2015) Involvement of neuroactive steroids in pain, depression and anxiety Mod Trends Pharmacopsychiatri 30, 94-102.

Mikuls TR, Weaver AL (2003) Lessons learned in the use of tumor necrosis factor-alpha inhibitors in the treatment of rheumatoid arthritis. Curr Rheumatol Rep 5:270–277 Available at: http://www.ncbi.nlm.nih.gov/pubmed/14531954 [Accessed June 22, 2018].

Milin M, Cornec D, Chastaing M, Griner V, Berrouiguet S, Nowak E, Marhadour T, Saraux A, Devauchelle-Pensec V (2016) Sicca symptoms are associated with similar fatigue, anxiety, depression, and quality-of-life impairments in patients with and without primary Sjögren's syndrome. Jt Bone Spine 83:681–685 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1297319X15003127 [Accessed February 8, 2018].

Miller E, Walczak A, Majsterek I, Kędziora J (2013) Melatonin reduces oxidative stress in the erythrocytes of multiple sclerosis patients with secondary progressive clinical course. J Neuroimmunol 257:97–101 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165572813000507 [Accessed February 8, 2018].

Miller FW, Alfredsson L, Costenbader KH, Kamen DL, Nelson LM, Norris JM, De Roos AJ (2012) Epidemiology of environmental exposures and human autoimmune diseases: Findings from a National Institute of Environmental Health Sciences Expert Panel Workshop. J Autoimmun 39:259–271 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896841112000595 [Accessed February 8, 2018].

Mitsuoka K, Kikutani T, Miwa Y, Sato I (2018) Expression of CGRP neurotransmitter and vascular genesis marker mRNA is age-dependent in superior cervical ganglia of senescence-accelerated prone mice. Neurosci Lett 664:144–151 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29154859 [Accessed June 5, 2018].

Moalem-Taylor G, Allbutt HN, Iordanova MD, Tracey DJ (2007) Pain hypersensitivity in rats with experimental autoimmune neuritis, an animal model of human inflammatory demyelinating neuropathy. Brain Behav Immun 21:699–710 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0889159106002595 [Accessed June 7, 2018].

Moalem G, Xu K, Yu L (2004) T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. Neuroscience 129:767–777 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452204007407 [Accessed June 7, 2018].

Mogil JS (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nat Rev Neurosci 13:859–866 Available at: http://www.nature.com/articles/nrn3360 [Accessed February 8, 2018].

Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KVS, Lariviere WR, Groce MK,

Wallace MR, Kaplan L, Staud R, Ness TJ, Glover TL, Stankova M, Mayorov A, Hruby VJ, Grisel JE, Fillingim RB (2003) The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. Proc Natl Acad Sci U S A 100:4867–4872 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12663858 [Accessed February 8, 2018].

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22:6724–6731 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12151551 [Accessed February 8, 2018].

Moreira I, Teixeira F, Martins Silva A, Vasconcelos C, Farinha F, Santos E (2015) Frequent involvement of central nervous system in primary Sjögren syndrome. Rheumatol Int 35:289–294 Available at: http://link.springer.com/10.1007/s00296-014-3097-9 [Accessed February 8, 2018].

Moriarty O, McGuire BE, Finn DP (2011) The effect of pain on cognitive function: A review of clinical and preclinical research. Prog Neurobiol 93:385–404 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21216272 [Accessed April 30, 2018].

Morrison G, Van Langenberg DR, Gibson SJ, Gibson PR (2013) Chronic pain in inflammatory bowel disease. Inflamm Bowel Dis 19:1210–1217 Available at: https://academic.oup.com/ibdjournal/article/19/6/1210-1217/4603112 [Accessed February 8, 2018].

Moses-Kolko EL, Price JC, Shah N, Berga S, Sereika SM, Fisher PM, Coleman R, Becker C, Mason NS, Loucks T, Meltzer CC (2011) Age, sex and reproductive hormone effects on brain serotonin-1A and serotonin-2A receptor binding in a healthy population. Neuropsychopharmacology 36:2729–2740 Available at: http://www.nature.com/articles/npp2011163 [Accessed February 8, 2018].

Moudgil KD (2015) Interplay among cytokines and T cell subsets in the progression and control of immune-mediated diseases. Cytokine 74:1–4 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26026376 [Accessed June 7, 2018].

Moulin DE, Clark AJ, Speechley M, Morley-Forster PK (2002) Chronic pain in Canada--prevalence, treatment, impact and the role of opioid analgesia. Pain Res Manag 7:179–184 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12518174 [Accessed April 30, 2018].

Moynes DM, Vanner SJ, Lomax AE (2014) Participation of interleukin 17A in neuroimmune interactions. Brain Behav Immun 41:1–9 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0889159114000701 [Accessed February 8, 2018].

Muñoz-Islas E, Vidal-Cantú GC, Bravo-Hernández M, Cervantes-Durán C, Quiñonez-Bastidas GN, Pineda-Farias JB, Barragán-Iglesias P, Granados-Soto V (2014) Spinal 5-HT5A receptors mediate 5-HT-induced antinociception in several pain models in rats. Pharmacol Biochem Behav 120:25–32 Available at: https://www.sciencedirect.com/science/article/pii/S0091305714000239?via%3Dihub [Accessed February 8, 2018].

Murphy KL, Bethea JR, Fischer R (2017) Neuropathic Pain in Multiple Sclerosis—Current Therapeutic Intervention and Future Treatment Perspectives. Codon Publications. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29261265 [Accessed May 15, 2018].

Murphy SF, Schaeffer AJ, Done J, Wong L, Bell-Cohn A, Roman K, Cashy J, Ohlhausen M, Thumbikat P (2015) IL17 Mediates Pelvic Pain in Experimental Autoimmune Prostatitis (EAP) Kim J, ed. PLoS One 10:e0125623 Available at: http://dx.plos.org/10.1371/journal.pone.0125623 [Accessed February 8, 2018].

Murtas C, Ghiggeri GM (2016) Membranous glomerulonephritis: histological and serological features to differentiate cancer-related and non-related forms. J Nephrol 29:469–478 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26810113 [Accessed June 8, 2018].

Musgrave T, Benson C, Wong G, Browne I, Tenorio G, Rauw G, Baker GB, Kerr BJ (2011a) The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE). Brain Behav Immun 25:1677–1688 Available at: https://www.sciencedirect.com/science/article/pii/S088915911100208X?via%3Dihub [Accessed February 8, 2018].

Musgrave T, Tenorio G, Rauw G, Baker GB, Kerr BJ (2011b) Tissue concentration changes of amino acids and biogenic amines in the central nervous system of mice with experimental autoimmune encephalomyelitis (EAE). Neurochem Int 59:28–38 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0197018611001239 [Accessed February 8, 2018].

Nag S, Mokha SS (2014) Activation of a Gq-coupled membrane estrogen receptor rapidly attenuates α2-adrenoceptor-induced antinociception via an ERK I/II-dependent, non-genomic mechanism in the female rat. Neuroscience 267:122–134 Available at: https://www.sciencedirect.com/science/article/pii/S0306452214001523?via%3Dihub [Accessed February 8, 2018].

Nielsen CS, Knudsen GP, Steingrímsdóttir ÓA (2012) Twin studies of pain. Clin Genet 82:331–340 Available at: http://doi.wiley.com/10.1111/j.1399-0004.2012.01938.x [Accessed June 6, 2018].

Nikić I, Merkler D, Sorbara C, Brinkoetter M, Kreutzfeldt M, Bareyre FM, Brück W, Bishop D, Misgeld T, Kerschensteiner M (2011) A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat Med 17:495–499 Available at: http://www.nature.com/articles/nm.2324 [Accessed June 13, 2018].

Noorbakhsh F, Baker GB, Power C (2014) Allopregnanolone and neuroinflammation: a focus on multiple sclerosis. Front Cell Neurosci 8:134 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24917787 [Accessed April 17, 2018].

Noorbakhsh F, Ellestad KK, Maingat F, Warren KG, Han MH, Steinman L, Baker GB, Power C (2011) Impaired neurosteroid synthesis in multiple sclerosis. Brain 134:2703–2721 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21908875 [Accessed April 17, 2018].

Noorbakhsh B, Julian L, Waubant E (2016) Fatigue and depression predict quality of life in

patients with early multiple sclerosis: a longitudinal study. Eur J Neurol 23:1482–1486 Available at: http://doi.wiley.com/10.1111/ene.13102 [Accessed February 8, 2018].

Nozawa K, Ikeda K, Satoh M, Reeves WH, Stewart CM, Li Y-C, Yen TJ, Rios RM, Takamori K, Ogawa H, Sekigawa I, Takasaki Y, Chan EKL (2009) Autoantibody to NA14 is an independent marker primarily for Sjogren's syndrome. Front Biosci (Landmark Ed 14:3733–3739 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19273306 [Accessed June 8, 2018].

O'Connor AB, Schwid SR, Herrmann DN, Markman JD, Dworkin RH (2008) Pain associated with multiple sclerosis: Systematic review and proposed classification. Pain 137:96–111 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17928147 [Accessed May 15, 2018].

O'Neill J, Brock C, Olesen AE, Andresen T, Nilsson M, Dickenson AH (2012) Unravelling the mystery of capsaicin: a tool to understand and treat pain. Pharmacol Rev 64:939–971 Available at: http://pharmrev.aspetjournals.org/cgi/doi/10.1124/pr.112.006163 [Accessed June 1, 2018].

O'Shea JJ, Ma A, Lipsky P (2002) Cytokines and autoimmunity. Nat Rev Immunol 2:37–45 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11905836 [Accessed June 7, 2018].

Ogbonna AC, Clark AK, Malcangio M (2015) Development of monosodium acetate-induced osteoarthritis and inflammatory pain in ageing mice. Age (Omaha) 37:54 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25971876 [Accessed June 5, 2018].

Ohsawa T, Miyatake T, Yuki N (1993) Anti-B-series ganglioside-recognizing autoantibodies in an acute sensory neuropathy patient cause cell death of rat dorsal root ganglion neurons. Neurosci Lett 157:167–170 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8233048 [Accessed June 8, 2018].

Okie S (2010) A flood of opioids, a rising tide of deaths. N Engl J Med 363:1981–1985 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21083382 [Accessed June 7, 2018].

Olechowski CJ, Parmar A, Miller B, Stephan J, Tenorio G, Tran K, Leighton J, Kerr BJ (2010) A diminished response to formalin stimulation reveals a role for the glutamate transporters in the altered pain sensitivity of mice with experimental autoimmune encephalomyelitis (EAE). Pain 149:565–572 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20399559 [Accessed June 8, 2018].

Olechowski CJ, Tenorio G, Sauve Y, Kerr BJ (2013) Changes in nociceptive sensitivity and object recognition in experimental autoimmune encephalomyelitis (EAE). Exp Neurol 241:113–121 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23291347 [Accessed June 8, 2018].

Olechowski CJ, Truong JJ, Kerr BJ (2009) Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE). Pain 141:156–164 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-200901000-00025 [Accessed February 8, 2018].

Orefice N, Carotenuto A, Mangone G, Bues B, Rehm R, Cerillo I, Saccà F, Calignano A, Orefice G (2016) Assessment of neuroactive steroids in cerebrospinal fluid comparing acute relapse and stable disease in relapsing-remitting multiple sclerosis. J Steroid Biochem Mol Biol 159:1–7 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0960076016300267?via%3Dihub [Accessed April 4, 2018].

Orton S-M, Herrera BM, Yee IM, Valdar W, Ramagopalan S V, Sadovnick AD, Ebers GC (2006) Sex ratio of multiple sclerosis in Canada: a longitudinal study. neurology.thelancet.com 5 Available at: https://ac-els-cdn-com.login.ezproxy.library.ualberta.ca/S1474442206705816/1-s2.0-S1474442206705816-main.pdf?_tid=04d6de7d-1996-4d35-8c41-e13b428ac534&acdnat=1526429066_99a18db567784e1ae7d084fa7441823c [Accessed May 15, 2018].

Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y (2016) Sex-based differences in autoimmune diseases. Ann Ist Super Sanita 52:205–212 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27364395 [Accessed February 8, 2018].

Osborne D, Sobczyńska-Malefora A (2015) Autoimmune mechanisms in pernicious anaemia & thyroid disease. Autoimmun Rev 14:763–768 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25936607 [Accessed June 8, 2018].

Österberg A, Boivie J, Thuomas K-Å (2005) Central pain in multiple sclerosis - prevalence and clinical characteristics. Eur J Pain 9:531–531 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16139182 [Accessed June 7, 2018].

Packman CH (2015) The clinical pictures of autoimmune hemolytic anemia. Transfus Med Hemotherapy 42:317–324 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26696800 [Accessed June 8, 2018].

Palazzo E, Genovese R, Mariani L, Siniscalco D, Marabese I, De Novellis V, Rossi F, Maione S (2004) Metabotropic glutamate receptor 5 and dorsal raphe serotonin release in inflammatory pain in rat. Eur J Pharmacol 492:169–176.

Palle P, Monaghan KL, Milne SM, Wan ECK (2017) Cytokine signaling in multiple sclerosis and its therapeutic applications. Med Sci 5:23 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29099039 [Accessed June 13, 2018].

Panitch H, Haley A, Hirsch R, Johnson K (1987) Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet 329:893–895 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0140673687928637 [Accessed June 13, 2018].

Patel DI, White LJ (2013) Effect of 10-day forced treadmill training on neurotrophic factors in experimental autoimmune encephalomyelitis. Appl Physiol Nutr Metab 38:194–199 Available at: http://www.nrcresearchpress.com/doi/10.1139/apnm-2012-0303 [Accessed February 8, 2018].

Pender MP (1986) Ascending impairment of nociception in rats with experimental allergic

encephalomyelitis. J Neurol Sci 75:317–328 Available at: http://www.ncbi.nlm.nih.gov/pubmed/3490542 [Accessed June 8, 2018].

Pertovaara A (2006) Noradrenergic pain modulation. Prog Neurobiol 80:53–83 Available at: https://www.sciencedirect.com/science/article/pii/S0301008206001067?via%3Dihub [Accessed February 8, 2018].

Pillai S, Mattoo H, Cariappa A (2011) B cells and autoimmunity. Curr Opin Immunol 23:721–731 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0952791511001531 [Accessed February 8, 2018].

Polachini CRN, Spanevello RM, Zanini D, Baldissarelli J, Pereira LB, Schetinger MRC, da Cruz IBM, Assmann CE, Bagatini MD, Morsch VM (2016) Evaluation of Delta-Aminolevulinic Dehydratase Activity, Oxidative Stress Biomarkers, and Vitamin D Levels in Patients with Multiple Sclerosis. Neurotox Res 29:230–242 Available at: http://link.springer.com/10.1007/s12640-015-9584-2 [Accessed February 8, 2018].

Pöllmann W, Feneberg W (2008) Current management of pain associated with multiple sclerosis. CNS Drugs 22:291–324 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18336059 [Accessed June 7, 2018].

Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF (2003) The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol 66:1499–1503 Available at: http://www.ncbi.nlm.nih.gov/pubmed/14555227 [Accessed February 8, 2018].

Portenoy RK, Ahmed E (2018) Cancer Pain Syndromes. Hematol Oncol Clin North Am 32:371–386 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0889858818300121?via%3Dihub [Accessed June 1, 2018].

Potter LE, Doolen S, Mifflin K, Tenorio G, Baker G, Taylor BK, Kerr BJ (2018) Antinociceptive Effects of the Antidepressant Phenelzine are Mediated by Context-Dependent Inhibition of Neuronal Responses in the Dorsal Horn. Neuroscience 383.

Probyn K, Bowers H, Mistry D, Caldwell F, Underwood M, Patel S, Sandhu HK, Matharu M, Pincus T, CHESS team. (2017) Non-pharmacological self-management for people living with migraine or tension-type headache: a systematic review including analysis of intervention components. BMJ Open 7:e016670 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28801425 [Accessed June 7, 2018].

Pryor WM, Freeman KG, Larson RD, Edwards GL, White LJ (2015) Chronic exercise confers neuroprotection in experimental autoimmune encephalomyelitis. J Neurosci Res 93:697–706 Available at: http://doi.wiley.com/10.1002/jnr.23528 [Accessed February 8, 2018].

Purdy RH, Morrow AL, Moore PH, Paul SM (1991) Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. Proc Natl Acad Sci U S A 88:4553–4557 Available at: http://www.ncbi.nlm.nih.gov/pubmed/1852011 [Accessed April 13, 2018].

Quick ML, Mukherjee S, Rudick CN, Done JD, Schaeffer AJ, Thumbikat P (2012) CCL2 and CCL3 are essential mediators of pelvic pain in experimental autoimmune prostatitis. Am J Physiol Integr Comp Physiol 303:R580–R589 Available at: http://www.physiology.org/doi/10.1152/ajpregu.00240.2012 [Accessed February 8, 2018].

Racine M et al. (2016) The impact of pain and itch on functioning and health-related quality of life in systemic sclerosis: An exploratory study. J Pain Symptom Manage 52:43–53 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0885392416000506 [Accessed June 8, 2018].

Radhakrishnan R, Moore SA, Sluka KA (2003) Unilateral carrageenan injection into muscle or joint induces chronic bilateral hyperalgesia in rats. Pain 104:567–577 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12927629 [Accessed June 1, 2018].

Rahn EJ, Iannitti T, Donahue RR, Taylor BK (2014) Sex differences in a mouse model of multiple sclerosis: neuropathic pain behavior in females but not males and protection from neurological deficits during proestrus. Biol Sex Differ 5:4 Available at: http://bsd.biomedcentral.com/articles/10.1186/2042-6410-5-4 [Accessed February 8, 2018].

Rajan B, Ahmed J, Shenoy N, Denny C, Ongole R, Binnal A (2014) Assessment of quality of life in patients with chronic oral mucosal diseases: a questionnaire-based study. Perm J 18:e123-7 Available at: http://www.thepermanentejournal.org/issues/2014/winter/5600-oral-mucosal-diseases.html [Accessed June 8, 2018].

Reichling DB, Levine JD (2011) Pain and death: Neurodegenerative disease mechanisms in the nociceptor. Ann Neurol 69:13–21 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21280072 [Accessed June 13, 2018].

Reimer M, Rempe T, Diedrichs C, Baron R, Gierthmühlen J (2016) Sensitization of the nociceptive system in complex regional pain syndrome. Sommer C, ed. PLoS One 11:e0154553 Available at: http://dx.plos.org/10.1371/journal.pone.0154553 [Accessed February 8, 2018].

Ren K, Dubner R (2010) Interactions between the immune and nervous systems in pain. Nat Med 16:1267–1276 Available at: http://www.nature.com/articles/nm.2234 [Accessed February 8, 2018].

Reynolds ER, Ashbaugh AD, Hockenberry BJ, McGrew CA (2018) Multiple sclerosis and exercise. Curr Sports Med Rep 17:31–35 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29315107 [Accessed May 15, 2018].

Rhee I, Veillette A (2012) Protein tyrosine phosphatases in lymphocyte activation and autoimmunity. Nat Immunol 13:439–447 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22513334 [Accessed June 8, 2018].

Richters L, Lange N, Renner R, Treiber N, Ghanem A, Tiemann K, Scharffetter-Kochanek K, Bloch W, Brixius K (2011) Exercise-induced adaptations of cardiac redox homeostasis and remodeling in heterozygous SOD2-knockout mice. J Appl Physiol 111:1431–1440 Available at: http://www.physiology.org/doi/10.1152/japplphysiol.01392.2010 [Accessed

February 8, 2018].

Riley JL, Robinson ME, Wise EA, Myers CD, Fillingim RB (1998) Sex differences in the perception of noxious experimental stimuli: a meta-analysis. Pain 74:181–187 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9520232 [Accessed February 8, 2018].

Rocha-González HI, Meneses A, Carlton SM, Granados-Soto V (2005) Pronociceptive role of peripheral and spinal 5-HT7 receptors in the formalin test. Pain 117:182–192 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16098671 [Accessed February 8, 2018].

Rodrigues DH, Leles BP, Costa VV, Miranda AS, Cisalpino D, Gomes DA, de Souza DG, Teixeira AL (2016) IL-1β Is involved with the generation of pain in experimental autoimmune encephalomyelitis. Mol Neurobiol 53:6540–6547 Available at: http://link.springer.com/10.1007/s12035-015-9552-0 [Accessed February 8, 2018].

Roh D-H, Kim H-W, Yoon S-Y, Kang S-Y, Kwon Y-B, Cho K-H, Han H-J, Ryu Y-H, Choi S-M, Lee H-J, Beitz AJ, Lee J-H (2006) Bee venom injection significantly reduces nociceptive behavior in the mouse formalin test via capsaicin-insensitive afferents. J Pain 7:500–512 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16814689 [Accessed February 8, 2018].

Romagnani S (2000) T-cell subsets (Th1 versus Th2). Ann Allergy, Asthma Immunol 85:9–21 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10923599 [Accessed June 7, 2018].

Rossi S, Furlan R, De Chiara V, Musella A, Lo Giudice T, Mataluni G, Cavasinni F, Cantarella C, Bernardi G, Muzio L, Martorana A, Martino G, Centonze D (2009) Exercise attenuates the clinical, synaptic and dendritic abnormalities of experimental autoimmune encephalomyelitis. Neurobiol Dis 36:51–59 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0969996109001636 [Accessed February 8, 2018].

Rottenberg Y, Jacobs JM, Stessman J (2015) Prevalence of pain with advancing age brief report. J Am Med Dir Assoc 16:264.e1-264.e5 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25659621 [Accessed June 5, 2018].

Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, Clark RB (1990) An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. J Exp Med 172:1193–1200 Available at: http://www.ncbi.nlm.nih.gov/pubmed/2212948 [Accessed June 13, 2018].

Rustagi T, Njei B (2015) Antioxidant therapy for pain reduction in patients with chronic pancreatitis. Pancreas 44:812–818 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25882696 [Accessed June 13, 2018].

Ruts L, van Doorn PA, Lombardi R, Haasdijk ED, Penza P, Tulen JHM, Hempel RJ, van den Meiracker AH, Lauria G (2012) Unmyelinated and myelinated skin nerve damage in Guillain–Barré syndrome: Correlation with pain and recovery. Pain 153:399–409 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-

201202000-00022 [Accessed February 8, 2018].

Saarto T, Wiffen PJ (2010) Antidepressants for neuropathic pain: a Cochrane review. J Neurol Neurosurg Psychiatry 81:1372–1373 Available at: http://jnnp.bmj.com/cgi/doi/10.1136/jnnp.2008.144964 [Accessed February 8, 2018].

Sagheddu C, Aroni S, De Felice M, Lecca S, Luchicchi A, Melis M, Muntoni AL, Romano R, Palazzo E, Guida F, Maione S, Pistis M (2015) Enhanced serotonin and mesolimbic dopamine transmissions in a rat model of neuropathic pain. Neuropharmacology 97:383–393 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390815002610 [Accessed February 8, 2018].

Salou M, Nicol B, Garcia A, Laplaud D-A (2015) Involvement of CD8+ T Cells in Multiple Sclerosis. Front Immunol 6 Available at: http://journal.frontiersin.org/Article/10.3389/fimmu.2015.00604/abstract [Accessed February 8, 2018].

Salter MW, Beggs S (2014) Sublime Microglia: Expanding Roles for the Guardians of the CNS. Cell 158:15–24 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0092867414007296 [Accessed February 8, 2018].

Samuels J, Ng Y-S, Coupillaud C, Paget D, Meffre E (2005) Impaired early B cell tolerance in patients with rheumatoid arthritis. J Exp Med 201:1659–1667 Available at: http://www.jem.org/lookup/doi/10.1084/jem.20042321 [Accessed June 8, 2018].

Sarkar S, Cooney LA, Fox DA (2010) The role of T helper type 17 cells in inflammatory arthritis. Clin Exp Immunol 159:225–237 Available at: http://doi.wiley.com/10.1111/j.1365-2249.2009.04016.x [Accessed February 8, 2018].

Savettieri G, Messina D, Andreoli V, Bonavita S, Caltagirone C, Cittadella R, Farina D, Fazio MC, Girlanda P, Le Pira F, Liguori M, Lugaresi A, Nocentini U, Reggio A, Salemi G, Tedeschi G, Trojano M, Valentino P, Quattrone A (2004) Gender-related effect of clinical and genetic variables on the cognitive impairment in multiple sclerosis. J Neurol 251:1208–1214 Available at: http://link.springer.com/10.1007/s00415-004-0508-y [Accessed April 13, 2018].

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–1212 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11368918 [Accessed February 8, 2018].

Schanberg LE, Anthony KK, Gil KM, Maurin EC (2003) Daily pain and symptoms in children with polyarticular arthritis. Arthritis Rheum 48:1390–1397 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12746912 [Accessed June 8, 2018].

Schmitz K, Pickert G, Wijnvoord N, Häussler A, Tegeder I (2013) Dichotomy of CCL21 and CXCR3 in nerve injury-evoked and autoimmunity-evoked hyperalgesia. Brain Behav Immun 32:186–200 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0889159113001797 [Accessed February 8,

2018].

Scholz J, Woolf CJ (2007) The neuropathic pain triad: Neurons, immune cells and glia. Nat Neurosci 10:1361–1368.

Schüle C, Eser D, Baghai TC, Nothdurfter C, Kessler JS, Rupprecht R (2011) Neuroactive steroids in affective disorders: target for novel antidepressant or anxiolytic drugs? Neuroscience 191:55–77 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0306452211002831 [Accessed April 17, 2018].

Schulte U, Thumfart J-O, Klöcker N, Sailer CA, Bildl W, Biniossek M, Dehn D, Deller T, Eble S, Abbass K, Wangler T, Knaus H-G, Fakler B (2006) The epilepsy-linked Lgi1 protein assembles into presynaptic Kv1 channels and inhibits inactivation by Kvbeta1. Neuron 49:697–706 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627306000900 [Accessed June 8, 2018].

Schütze R, Rees C, Smith A, Slater H, Campbell JM, O'Sullivan P (2018) How can we best reduce pain catastrophizing in adults with chronic noncancer pain? A Systematic Review and Meta-Analysis. J Pain 19:233–256 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29122652 [Accessed June 7, 2018].

Scioli-Salter E, Forman DE, Otis JD, Tun C, Allsup K, Marx CE, Hauger RL, Shipherd JC, Higgins D, Tyzik A, Rasmusson AM (2016) Potential neurobiological benefits of exercise in chronic pain and posttraumatic stress disorder: Pilot study. J Rehabil Res Dev 53:95–106 Available at: http://www.rehab.research.va.gov/jour/2016/531/pdf/JRRD-2014-10-0267.pdf [Accessed April 11, 2018].

Searle A, Spink M, Ho A, Chuter V (2015) Exercise interventions for the treatment of chronic low back pain: a systematic review and meta-analysis of randomised controlled trials. Clin Rehabil 29:1155–1167 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25681408 [Accessed June 7, 2018].

Seeling M, Hillenhoff U, David JP, Schett G, Tuckermann J, Lux A, Nimmerjahn F (2013) Inflammatory monocytes and Fc receptor IV on osteoclasts are critical for bone destruction during inflammatory arthritis in mice. Proc Natl Acad Sci 110:10729–10734 Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1301001110 [Accessed February 8, 2018].

Segal BM, Pogatchnik B, Henn L, Rudser K, Sivils KM (2013) Pain severity and neuropathic pain symptoms in primary Sjögren's syndrome: A comparison study of Sseropositive and seronegative Sjögren's syndrome patients. Arthritis Care Res (Hoboken) 65:1291–1298 Available at: http://doi.wiley.com/10.1002/acr.21956 [Accessed February 8, 2018].

Selmi C, Lu Q, Humble MC (2012) Heritability versus the role of the environment in autoimmunity. J Autoimmun 39:249–252 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896841112001072 [Accessed February 8, 2018].

Sexton JE, Cox JJ, Zhao J, Wood JN (2018) The genetics of pain: Implications for therapeutics.

Annu Rev Pharmacol Toxicol 58:123–142 Available at: http://www.annualreviews.org/doi/10.1146/annurev-pharmtox-010617-052554 [Accessed June 6, 2018].

Sharief MK, Hentges R (1991) Association between tumor necrosis factor-α and disease progression in patients with multiple sclerosis. N Engl J Med 325:467–472 Available at: http://www.ncbi.nlm.nih.gov/pubmed/1852181 [Accessed June 13, 2018].

Sherman RA, Sherman CJ, Parker L (1984) Chronic phantom and stump pain among american veterans: results of a survey. Pain 18:83–95.

Shirani A, Zhao Y, Kingwell E, Rieckmann P, Tremlett H (2012) Temporal trends of disability progression in multiple sclerosis: findings from British Columbia, Canada (1975-2009). Mult Scler 18:442–450 Available at: http://journals.sagepub.com/doi/10.1177/1352458511422097 [Accessed May 15, 2018].

Siddall PJ, Middleton JW (2015) Spinal cord injury-induced pain: mechanisms and treatments. Pain Manag 5:493–507 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26402151 [Accessed June 1, 2018].

Simone DA, Baumann TK, LaMotte RH (1989) Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin. Pain 38:99–107 Available at: http://www.ncbi.nlm.nih.gov/pubmed/2780068 [Accessed June 1, 2018].

Sloane E, Ledeboer A, Seibert W, Coats B, van Strien M, Maier SF, Johnson KW, Chavez R, Watkins LR, Leinwand L, Milligan ED, Van Dam AM (2009) Anti-inflammatory cytokine gene therapy decreases sensory and motor dysfunction in experimental Multiple Sclerosis: MOG-EAE behavioral and anatomical symptom treatment with cytokine gene therapy. Brain Behav Immun 23:92–100 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0889159108003474 [Accessed February 8, 2018].

Sluka KA, O'Donnell JM, Danielson J, Rasmussen LA (2013) Regular physical activity prevents development of chronic pain and activation of central neurons. J Appl Physiol 114:725–733 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23271699 [Accessed February 21, 2018].

Sokolove J, Pisetsky D (2016) Bone loss, pain and inflammation: three faces of ACPA in RA pathogenesis. Ann Rheum Dis 75:637–639 Available at: http://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2015-208308 [Accessed February 8, 2018].

Solaro C, Messmer Uccelli M (2011) Management of pain in multiple sclerosis: a pharmacological approach. Nat Rev Neurol 7:519–527 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21844896 [Accessed May 15, 2018].

Solleiro-Villavicencio H, Rivas-Arancibia S (2018) Effect of Chronic Oxidative Stress on Neuroinflammatory Response Mediated by CD4+T Cells in Neurodegenerative Diseases. Front Cell Neurosci 12:114 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29755324

[Accessed June 12, 2018].

Sommer C (2004a) Serotonin in pain and analgesia: Actions in the periphery. Mol Neurobiol 30:117–126 Available at: http://link.springer.com/10.1385/MN:30:2:117 [Accessed February 8, 2018].

Sommer C (2004b) Serotonin in pain and analgesia: actions in the periphery. Mol Neurobiol 30:117–125 Available at: http://link.springer.com/10.1385/MN:30:2:117 [Accessed February 8, 2018].

Sorge RE et al. (2012) Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. Nat Med 18:595–599 Available at: http://www.nature.com/articles/nm.2710 [Accessed February 8, 2018].

Sorge RE et al. (2014) Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 11:629–632 Available at: http://www.nature.com/articles/nmeth.2935 [Accessed February 8, 2018].

Sorge RE et al. (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nat Neurosci 18:1081–1083 Available at: http://www.nature.com/articles/nn.4053 [Accessed February 8, 2018].

Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin J-S, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS (2011) Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. J Neurosci 31:15450–15454 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22031891 [Accessed February 8, 2018].

Sospedra M, Martin R (2005) Immunology of multiple sclerosis. Annu Rev Immunol 23:683–747 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15771584 [Accessed June 7, 2018].

Sounvoravong S, Nakashima MN, Wada M, Nakashima K (2004) Decrease in serotonin concentration in raphe magnus nucleus and attenuation of morphine analgesia in two mice models of neuropathic pain. Eur J Pharmacol 484:217–223.

Souza PS, Gonçalves ED, Pedroso GS, Farias HR, Junqueira SC, Marcon R, Tuon T, Cola M, Silveira PCL, Santos AR, Calixto JB, Souza CT, de Pinho RA, Dutra RC (2017) Physical exercise attenuates experimental autoimmune encephalomyelitis by inhibiting peripheral immune response and blood-brain barrier disruption. Mol Neurobiol 54:4723–4737 Available at: http://link.springer.com/10.1007/s12035-016-0014-0 [Accessed February 8, 2018].

Spano F, Donovan JC (2015) Alopecia areata: Part 1: pathogenesis, diagnosis, and prognosis. Can Fam Physician 61:751–755 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26371097 [Accessed June 8, 2018].

Stahl SM, Porreca F, Taylor CP, Cheung R, Thorpe AJ, Clair A (2013) The diverse therapeutic actions of pregabalin: is a single mechanism responsible for several pharmacological activities? Trends Pharmacol Sci 34:332–339 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/23642658 [Accessed June 7, 2018].

Steed PM et al. (2003) Inactivation of TNF signaling by rationally designed dominant-negative TNF variants. Science 301:1895–1898 Available at: http://www.ncbi.nlm.nih.gov/pubmed/14512626 [Accessed June 22, 2018].

Steinman L (2010) Mixed results with modulation of TH-17 cells in human autoimmune diseases. Nat Immunol 11:41–44 Available at: http://www.nature.com/doifinder/10.1038/ni.1803 [Accessed February 8, 2018].

Sternberg WF, Ritchie J, Mogil JS (2004) Qualitative sex differences in kappa-opioid analgesia in mice are dependent on age. Neurosci Lett 363:178–181 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304394004004008 [Accessed February 8, 2018].

Sun C, Zhang J, Chen L, Liu T, Xu G, Li C, Yuan W, Xu H, Su Z (2017) IL-17 contributed to the neuropathic pain following peripheral nerve injury by promoting astrocyte proliferation and secretion of proinflammatory cytokines. Mol Med Rep 15:89–96 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27959414 [Accessed May 25, 2018].

Suurmeijer TP, Waltz M, Moum T, Guillemin F, van Sonderen FL, Briançon S, Sanderman R, van den Heuvel WJ (2001) Quality of life profiles in the first years of rheumatoid arthritis: results from the EURIDISS longitudinal study. Arthritis Rheum 45:111–121 Available at: http://doi.wiley.com/10.1002/1529-0131%28200104%2945%3A2%3C111%3A%3AAID-ANR162%3E3.0.CO%3B2-E [Accessed June 8, 2018].

Tajerian M, Clark JD (2016) New Concepts in Complex Regional Pain Syndrome. Hand Clin 32:41–49 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0749071215000992 [Accessed February 8, 2018].

Talukdar R, Murthy HVV, Reddy DN (2015) Role of methionine containing antioxidant combination in the management of pain in chronic pancreatitis: A systematic review and meta-analysis. Pancreatology 15:136–144 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25648074 [Accessed June 13, 2018].

Tamburin S, Borg K, Caro XJ, Jann S, Clark AJ, Magrinelli F, Sobue G, Werhagen L, Zanette G, Koike H, Späth PJ, Vincent A, Goebel A (2014) Immunoglobulin G for the Treatment of Chronic Pain: Report of an Expert Workshop. Pain Med 15:1072–1082 Available at: https://academic.oup.com/painmedicine/article-lookup/doi/10.1111/pme.12319 [Accessed February 8, 2018].

Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc Natl Acad Sci U S A 102:5856–5861 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=556308&tool=pmcentrez&rendertype=abstract.

Tashiro A, Bereiter DA, Thompson R, Nishida Y (2014) GABAergic influence on temporomandibular joint-responsive spinomedullary neurons depends on estrogen status.

Neuroscience 259:53–62 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452213010002 [Accessed February 8, 2018].

Tateiwa H, Kawano T, Nishigaki A, Yamanaka D, Aoyama B, Shigematsu-Locatelli M, Eguchi S, Locatelli FM, Yokoyama M (2018) The role of hippocampal brain-derived neurotrophic factor in age-related differences in neuropathic pain behavior in rats. Life Sci 197:56–66 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0024320518300420?via%3Dihub [Accessed June 5, 2018].

Tegeder I et al. (2006) GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence. Nat Med 12:1269–1277 Available at: http://www.nature.com/articles/nm1490 [Accessed June 6, 2018].

Tékus V, Hajna Z, Borbély É, Markovics A, Bagoly T, Szolcsányi J, Thompson V, Kemény Á, Helyes Z, Goebel A (2014) A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with complex regional pain syndrome. Pain 155:299–308 Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00006396-201402000-00013 [Accessed February 8, 2018].

ter Borg EJ, Kelder JC (2014) Lower prevalence of extra-glandular manifestations and anti-SSB antibodies in patients with primary Sjögren's syndrome and widespread pain: evidence for a relatively benign subset. Clin Exp Rheumatol 32:349–353 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24529195 [Accessed June 8, 2018].

Terkelsen AJ, Bach FW, Jensen TS (2008) Experimental Forearm Immobilization in Humans Induces Cold and Mechanical Hyperalgesia. Anesthesiology 109:297–307 Available at: http://anesthesiology.pubs.asahq.org/Article.aspx?doi=10.1097/ALN.0b013e31817f4c9d [Accessed February 8, 2018].

Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O (2018) Multiple sclerosis. Lancet 391:1622–1636 Available at: https://www-sciencedirect-com.login.ezproxy.library.ualberta.ca/science/article/pii/S0140673618304811?via%3Dihub [Accessed May 15, 2018].

Tian D, Tian M, Zhang L, Zhao P, Cui Y, Li J (2018) High fat diet sensitizes fibromyalgia-like pain behaviors in mice via tumor necrosis factor alpha Ahmad A, ed. PLoS One 13:e0190861 Available at: http://dx.plos.org/10.1371/journal.pone.0190861 [Accessed May 25, 2018].

Tomasson G et al. (2012) Assessment of health-related quality of life as an outcome measure in granulomatosis with polyangiitis (Wegener's). Arthritis Care Res (Hoboken) 64:273–279 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21954229 [Accessed June 8, 2018].

Trang T, Al-Hasani R, Salvemini D, Salter MW, Gutstein H, Cahill CM (2015) Pain and Poppies: The Good, the Bad, and the Ugly of Opioid Analgesics. J Neurosci 35:13879–13888 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26468188 [Accessed June 7,

2018].

Trang T, Beggs S, Wan X, Salter MW (2009) P2X4-Receptor-Mediated Synthesis and Release of Brain-Derived Neurotrophic Factor in Microglia Is Dependent on Calcium and p38-Mitogen-Activated Protein Kinase Activation. J Neurosci 29:3518–3528 Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.5714-08.2009 [Accessed February 8, 2018].

Trojano M et al. (2012) Geographical variations in sex ratio trends over time in multiple sclerosis. Paul F, ed. PLoS One 7:e48078 Available at: http://dx.plos.org/10.1371/journal.pone.0048078 [Accessed February 8, 2018].

Tsuda M (2017) P2 receptors, microglial cytokines and chemokines, and neuropathic pain. J Neurosci Res 95:1319–1329 Available at: http://doi.wiley.com/10.1002/jnr.23816 [Accessed February 8, 2018].

Tsuda M, Inoue K (2016) Neuron-microglia interaction by purinergic signaling in neuropathic pain following neurodegeneration. Neuropharmacology 104:76–81 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390815300873 [Accessed June 7, 2018].

Tuem KB, Atey TM (2017) Neuroactive Steroids: Receptor Interactions and Responses. Front Neurol 8:442 Available at: http://journal.frontiersin.org/article/10.3389/fneur.2017.00442/full [Accessed April 4, 2018].

Veldman PH, Reynen HM, Arntz IE, Goris RJ (1993) Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. Lancet (London, England) 342:1012–1016 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8105263 [Accessed June 8, 2018].

Venasse M, Edwards T, Pilutti LA (2018) Exploring Wellness Interventions in Progressive Multiple Sclerosis: an Evidence-Based Review. Curr Treat Options Neurol 20:13 Available at: http://link.springer.com/10.1007/s11940-018-0497-2 [Accessed May 15, 2018].

Verma V, Sheikh Z, Ahmed AS (2015) Nociception and role of immune system in pain. Acta Neurol Belg 115:213–220 Available at: http://link.springer.com/10.1007/s13760-014-0411-y [Accessed February 8, 2018].

Vincler M, Maixner W, Vierck CJ, Light AR (n.d.) Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin. Pharmacol Biochem Behav 69:315–324 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11509187 [Accessed February 8, 2018].

Vitali C, Del Papa N (2015) Pain in primary Sjögren's syndrome. Best Pract Res Clin Rheumatol 29:63–70 Available at: http://linkinghub.elsevier.com/retrieve/pii/S1521694215000406 [Accessed February 8, 2018].

Voskuhl RR, Sawalha AH, Itoh Y (2018) Sex chromosome contributions to sex differences in multiple sclerosis susceptibility and progression. Mult Scler J 24:22–31 Available at: http://journals.sagepub.com/doi/10.1177/1352458517737394 [Accessed May 15, 2018].

Wang M, He Y, Eisenman LN, Fields C, Zeng C-M, Mathews J, Benz A, Fu T, Zorumski E, Steinbach JH, Covey DF, Zorumski CF, Mennerick S (2002) 3beta -hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists. J Neurosci 22:3366–3375 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11978813 [Accessed April 17, 2018].

Wang P, Xie K, Wang C, Bi J (2014) Oxidative stress induced by lipid peroxidation is related with inflammation of demyelination and neurodegeneration in multiple sclerosis. Eur Neurol 72:249–254 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25277682 [Accessed June 12, 2018].

Wang Y, Feng C, He H, He J, Wang J, Li X, Wang S, Li W, Hou J, Liu T, Fang D, Xie S (2018) Sensitization of TRPV1 receptors by TNF-α orchestrates the development of vincristine-induced pain. Oncol Lett 15:5013–5019 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29552137 [Accessed May 25, 2018].

Wen C, Xu M, Mo C, Cheng Z, Guo Q, Zhu X (2018) JMJD6 exerts function in neuropathic pain by regulating NF-κB following peripheral nerve injury in rats. Int J Mol Med 42:633–642 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29620141 [Accessed May 25, 2018].

Wichmann MW, Zellweger R, DeMaso CM, Ayala A, Chaudry IH (1996) Mechanism of Immunosuppression in Males Following Trauma-Hemorrhage. Arch Surg 131:1186 Available at: http://archsurg.jamanetwork.com/article.aspx?doi=10.1001/archsurg.1996.01430230068012 [Accessed February 8, 2018].

Wigerblad G et al. (2016) Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. Ann Rheum Dis 75:730–738 Available at: http://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2015-208094 [Accessed February 8, 2018].

Wimbiscus M, Kostenko O, Malone D (2010) MAO inhibitors: Risks, benefits, and lore. Cleve Clin J Med 77:859–882 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21147941 [Accessed June 14, 2018].

Woolf CJ (1996) Windup and central sensitization are not equivalent. Pain 66:105–108.

Wu S, Zhu M, Wang W, Wang Y, Li Y, Yew DT (2001) Changes of the expression of 5-HT receptor subtype mRNAs in rat dorsal root ganglion by complete Freund's adjuvant-induced in¯ammation. Neurosci Lett 307:183–186.

Xiao WH, Yu AL, Sorkin LS (1997) Electrophysiological characteristics of primary afferent fibers after systemic administration of anti-GD2 ganglioside antibody. Pain 69:145–151 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9060025 [Accessed June 8, 2018].

Xu M, Kontinen VK, Kalso E (1999) Endogenous noradrenergic tone controls symptoms of allodynia in the spinal nerve ligation model of neuropathic pain. Eur J Pharmacol 366:41–45 Available at: https://www.sciencedirect.com/science/article/pii/S0014299998009108?via%3Dihub

[Accessed February 9, 2018].

Yakes FM, Van Houten B (1997) Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A 94:514–519 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9012815 [Accessed June 13, 2018].

Yang H-W, Zhou L-J, Hu N-W, Xin W-J, Liu X-G (2005) Activation of Spinal D1/D5 Receptors Induces Late-Phase LTP of C-Fiber–Evoked Field Potentials in Rat Spinal Dorsal Horn. J Neurophysiol 94:961–967 Available at: http://www.physiology.org/doi/10.1152/jn.01324.2004 [Accessed February 8, 2018].

Yang M, Peyret C, Shi XQ, Siron N, Jang JH, Wu S, Fournier S, Zhang J (2015) Evidence from Human and Animal Studies: Pathological Roles of CD8+ T Cells in Autoimmune Peripheral Neuropathies. Front Immunol 6 Available at: http://journal.frontiersin.org/Article/10.3389/fimmu.2015.00532/abstract [Accessed February 8, 2018].

Yang M, Rainone A, Shi XQ, Fournier S, Zhang J (2014) A new animal model of spontaneous autoimmune peripheral polyneuropathy: implications for Guillain-Barré syndrome. Acta Neuropathol Commun 2:5 Available at: http://actaneurocomms.biomedcentral.com/articles/10.1186/2051-5960-2-5 [Accessed February 8, 2018].

Yao C, Weng Z, Zhang J, Feng T, Lin Y, Yao S (2016) Interleukin-17A Acts to Maintain Neuropathic Pain Through Activation of CaMKII/CREB Signaling in Spinal Neurons. Mol Neurobiol 53:3914–3926 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26166359 [Accessed May 25, 2018].

Yezierski RP, Hansson P (2018) Inflammatory and Neuropathic Pain From Bench to Bedside: What Went Wrong? J Pain 19:571–588 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29307749 [Accessed June 5, 2018].

Yuki N, Hartung H-P (2012) Guillain–Barré Syndrome. N Engl J Med 366:2294–2304 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22694000 [Accessed June 8, 2018].

Yurasov S, Tiller T, Tsuiji M, Velinzon K, Pascual V, Wardemann H, Nussenzweig MC (2006) Persistent expression of autoantibodies in SLE patients in remission. J Exp Med 203:2255–2261 Available at: http://www.jem.org/lookup/doi/10.1084/jem.20061446 [Accessed June 8, 2018].

Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, Nussenzweig MC (2005) Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med 201:703–711 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15738055 [Accessed June 8, 2018].

Zargari M, Allameh A, Sanati MH, Tiraihi T, Lavasani S, Emadyan O (2007) Relationship between the clinical scoring and demyelination in central nervous system with total antioxidant capacity of plasma during experimental autoimmune encephalomyelitis

development in mice. Neurosci Lett 412:24–28 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17157437 [Accessed February 8, 2018].

Zeilhofer HU, Möhler H, Di Lio A (2009) GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Trends Pharmacol Sci 30:397–402 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19616317 [Accessed February 8, 2018].

Zhang MA, Ahn JJ, Zhao FL, Selvanantham T, Mallevaey T, Stock N, Correa L, Clark R, Spaner D, Dunn SE (2015) Antagonizing Peroxisome Proliferator-Activated Receptor Activity Selectively Enhances Th1 Immunity in Male Mice. J Immunol 195:5189–5202 Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500449 [Accessed February 8, 2018].

Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, Akkermann R, Stanczyk FZ, Prat A, Steinman L, Dunn SE (2012a) Peroxisome proliferator-activated receptor (PPAR) and - regulate IFN and IL-17A production by human T cells in a sex-specific way. Proc Natl Acad Sci 109:9505–9510 Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1118458109 [Accessed February 8, 2018].

Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, Akkermann R, Stanczyk FZ, Prat A, Steinman L, Dunn SE (2012b) Peroxisome proliferator-activated receptor (PPAR)α and -γ regulate IFNγ and IL-17A production by human T cells in a sex-specific way. Proc Natl Acad Sci U S A 109:9505–9510 Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1118458109 [Accessed February 8, 2018].

Zhang YQ, Gao X, Zhang LM, Wu GC (2000) The release of serotonin in rat spinal dorsal horn and periaqueductal gray following carrageenan inflammation. Neuroreport 11:3539–3543 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11095514 [Accessed May 1, 2018].

Zhang Z, Wang X, Wang W, Lu Y-G, Pan ZZ (2013) Brain-Derived Neurotrophic Factor-Mediated Downregulation of Brainstem K+-Cl- Cotransporter and Cell-Type-Specific GABA Impairment for Activation of Descending Pain Facilitation. Mol Pharmacol 84:511–520 Available at: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.113.086496.

Zhang Z, Zhang Z-Y, Fauser U, Schluesener HJ (2008) Mechanical allodynia and spinal up-regulation of P2X4 receptor in experimental autoimmune neuritis rats. Neuroscience 152:495–501 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452207016685 [Accessed February 8, 2018].

Zhao X, Xu Y, Zhao Q, Chen C-R, Liu A-M, Huang Z-L (2012) Curcumin exerts antinociceptive effects in a mouse model of neuropathic pain: descending monoamine system and opioid receptors are differentially involved. Neuropharmacology 62:843–854 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390811004072 [Accessed February 8, 2018].

Zhao X, Yu C, Wang C, Zhang J-F, Zhou W-H, Cui W-G, Ye F, Xu Y (2014) Chronic resveratrol treatment exerts antihyperalgesic effect and corrects co-morbid depressive like behaviors in mice with mononeuropathy: involvement of serotonergic system. Neuropharmacology 85:131–141 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390814001579 [Accessed February 8,

2018].

Zhi L, Ustyugova I V, Chen X, Zhang Q, Wu MX (2012) Enhanced Th17 differentiation and aggravated arthritis in IEX-1-deficient mice by mitochondrial reactive oxygen species-mediated signaling. J Immunol 189:1639–1647 Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200528 [Accessed June 12, 2018].

Zhu W, Acosta C, MacNeil B, Cortes C, Intrater H, Gong Y, Namaka M (2013) Elevated Expression of Fractalkine (CX3CL1) and Fractalkine Receptor (CX3CR1) in the Dorsal Root Ganglia and Spinal Cord in Experimental Autoimmune Encephalomyelitis: Implications in Multiple Sclerosis-Induced Neuropathic Pain. Biomed Res Int 2013:1–14 Available at: http://www.hindawi.com/journals/bmri/2013/480702/ [Accessed February 8, 2018].