Investigating the Effects of N^2-Acetylphenelzine in the Sexes in Experimental Autoimmune Encephalomyelitis

by

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Abstract

Chronic pain is a highly prevalent symptom in Multiple Sclerosis (MS) and affects approximately half of patients at some stage of the disease. MS affects women more frequently than men and neuropathic pain is also reported as more severe, and frequent, in females with the disease. The underlying causes of neuropathic pain remain to be elucidated and it is unclear why females are more greatly affected. The animal model experimental autoimmune encephalomyelitis (EAE) is used to study the pathophysiology of MS and MS-related pain. Studies have shown that pain behaviours and the disease course in mice with EAE may be improved with the administration of the antidepressant phenelzine (PLZ) which acts to elevate noradrenaline (NA), serotonin (5-HT), and gamma-aminobutyric acid (GABA) levels. Interestingly, studies have also demonstrated significant sex differences in the behavioural responses to PLZ and an acetylated derivative, N^2-acetylphenelzine (N2-Ac-PLZ) (that elevates NA and 5-HT, but not GABA, levels) in the formalin pain assay. We have found that female mice do not respond to N2-Ac-PLZ in this particular pain model. As such, I sought to examine whether N2-Ac-PLZ beneficially improves pain behaviours and disease course within the sexes in mice with EAE.

C57/BL6 mice of both sexes were treated with myelin oligodendrocytes glycoprotein 35-55 (MOG_{35-55}) in Complete Freund's Adjuvant (CFA) and pertussis toxin to induce EAE. The mice were monitored daily, and starting on day 7 post-induction, chronically treated with a vehicle or N2-Ac-PLZ every other day until day 34 post-induction. I used behavioural tasks to assess exploratory, anxiety-like, and mechanical hypersensitivity behaviours in the mice, as well as high performance liquid chromatography (HPLC) to assess spinal levels of NA, 5-HT, and GABA.

In contrast to the formalin model, I find that N2-Ac-PLZ improved tactile hypersensitivity only in female mice with EAE. N2-Ac-PLZ treatment had no effect on the disease course of female or male mice with EAE. My examination of neurotransmitter levels within the spinal cord revealed that N2-Ac-PLZ treatment acted to increase levels of NA and 5-HT in both females and males, but with higher elevations observed in females.

This study shows that treatment with N2-Ac-PLZ can attenuate tactile hypersensitivity in a disease-related chronic pain paradigm in a sex-specific manner. These data provide further evidence of sex differences in EAE, and insight into the causes of pain in the disease.

Preface

This research was conducted in collaboration with Dr. Bradley Kerr and Dr. Glen Baker at the University of Alberta. Dr. Kerr's lab induced the mouse model of EAE used in the study. After the mice were euthanized and tissue was removed, all samples were processed with high-performance liquid chromatography (HPLC) in co-operation with Dr. Baker's lab at the University of Alberta where I conducted my work. My work included clinical monitoring, behavioural measures, tissue processing, all HPLC, and subsequent data analysis. The thesis was written entirely by myself, with edits suggested by an examining committee. The project was conducted under ethical approval of the University of Alberta Animal Research Ethics Board.

Acknowledgments

First and foremost, I want to thank my supervisor Dr. Bradley Kerr, for taking a chance on a psychology student with little knowledge of Neuroscience. Your compassion and constant motivation were a blessing through the toughest times of my degree. Thank you for being the type of mentor a grad student could only dream to have. Thank you to my colleagues past and present: Kevin, Saad, Ana, Curtis, Kasia, Liam, Gustavo, and especially Katherine, who managed to guide me through the last two years by imparting her vast wisdom and somehow maintaining my (relative) saneness. Being in the Kerr Lab was an incredibly wonderful way to spend the last two years, and I am so grateful to everyone in it.

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Glossary of Terms & Abbreviations

Multiple Sclerosis: MS

Experimental Autoimmune Encephalomyelitis: EAE

Noradrenaline: NA

Serotonin: 5-HT

Gamma-aminobutyric acid: GABA

Myelin Oligodendrocytes Glycoprotein: MOG

Complete Freund's Adjuvant: CFA

Von-Frey Hairs: VF

Central Nervous System: CNS

Phenelzine: PLZ

N^2-Acetylphenelzine: N2-Ac-PLZ

Elevated Plus Maze: EPM

High Performance Liquid Chromatography: HPLC

1. Introduction

Chronic pain is a prevalent disorder that can be difficult to diagnose due to varying definitions. It can be characterized as any pain lasting longer than three months (Elliott, Smith, Penny, Smith & Chambers, 1999). Up to 30% of people are reported to suffer from chronic pain, but prevalence rates range widely due to variations in the diagnostic criteria (Benson et al., 2015; Breivik, Collett, Ventafridda, Cohen & Gallacher, 2006; Dansie & Turk, 2013; Elliott et al., 1999). Chronic pain is a primary disorder in itself but is also a secondary symptom of primary autoimmune diseases such as Rheumatoid Arthritis and Multiple Sclerosis (MS) (Mifflin & Kerr, 2016; Pöllmann & Feneberg, 2008). Many MS patients report experiencing chronic pain, such as headaches and back pain, at some point in their disease course (Pöllmann & Feneberg, 2008).

1.1 MS & Sex Differences

The incidence of MS is much higher in females, who also report higher levels of neuropathic pain, which is defined as "pain caused by a lesion or disease of the somatosensory system" (Merskey & Bogduk, 2011; Orton et al., 2006). MS is a neurodegenerative autoimmune disease that causes significant decline in cognitive, motor, sensory, and affective domains (Compston & Coles, 2008). The pathophysiology includes white matter abnormalities that can be detected by magnetic resonance imaging (MRI), demyelination, and axonal degeneration, all of which can contribute to the symptoms of MS. Chronic pain is a prevalent, and debilitating, symptom of MS that is reported to affect approximately 43% of patients at some point in their disease (Solaro et al., 2004). The appearance of MS is heterogeneous, with patients presenting as relapsing remitting (RRMS), progressive (PMS), or progressive relapsing MS as outlined by the Multiple Sclerosis Society of Canada (MSSC). This heterogeneity within the clinical population makes the treatment of MS all the more difficult, since not all patients will respond to available

treatments. Many sex differences have been reported in MS, but the main one is that females have a higher incidence of MS, as well as reported pain (Bove & Chitnis, 2014; Orton et al., 2006). There have been multiple theories as to why females are affected more than males, such as the presence of reproductive hormones, but the mechanisms underlying sex differences in MS remain to be revealed (Bove & Chitnis, 2014). Heterogeneity and sex differences within the clinical population are important factors when considering the development and testing of new treatments in order to more efficiently treat the disease or the pain associated with it (Solaro, Trabucco & Messmer Uccelli, 2013).

1.2 Experimental Autoimmune Encephalomyelitis (EAE) & Pain

The animal model most commonly used to study MS, experimental autoimmune encephalomyelitis (EAE), is used in order to investigate the underlying mechanisms of pain in MS, as well as to develop new treatments (Dray, 1995). EAE is a disease model that mimics both the pathophysiology and symptoms of pain hypersensitivity in MS, which allows researchers to effectively test and improve treatments that could beneficially affect the clinical population (Aharoni, 2013; Olechowski, Truong & Kerr, 2009). There are multiple ways to induce EAE, but the Kerr laboratory works solely with myelin oligodendrocytes glycoprotein 35-55 (MOG_{35-55}) in Complete Freund's Adjuvant (CFA), and pertussis toxin (PT) (Benson, Wong, Tenorio, Baker & Kerr, 2013). The MOG_{35-55} and CFA are administered via subcutaneous injections which are followed by two intraperitoneal injections of PT (Benson et al., 2013; Bittner, Afzali, Wiendl & Meuth, 2014; Hofstetter, Shive & Forsthuber, 2002). PT facilitates EAE induction and increases disease incidence and severity, primarily through its ability to facilitate the break-down of the blood-brain barrier (BBB) (Linthicum, Munoz & Blaskett, 1982). The addition of CFA alongside PT acts to accelerate the animals' immune response to the mildly foreign MOG peptide, which is

similar to the mouse's myelin peptide, and is attacked by their immune system. This specific model of EAE mimics a chronic progressive disease course often seen in MS patients (Robinson, Harp, Noronha & Miller, 2014).

Neuropathic pain can be measured in EAE by using standard behavioural assays to assess sensitivity to mechanical and thermal stimuli (Olechowski et al., 2009). At disease onset (the first day that mice show clinical signs of EAE; clinical grade 1), thresholds to Von Frey (VF) hairs were reduced, indicating an increased sensitivity to mechanical stimuli. Mice with EAE also exhibit prolonged responses following the application of innocuous acetone to the hind paws in the acetone test (Olechowski et al., 2009; Potter et al., 2016). Olechowski and colleagues (2009) also determined immunohistologically that mice with EAE who demonstrated enhanced nociceptive behaviors at disease onset had increased inflammation in the superficial dorsal horn of the spinal cord including the presence of $CD3^+$ T-cells and reactive gliosis (enhanced expression of the F4/80 antigen on microglia/macrophages).

Neurotransmitters like noradrenaline (NA), serotonin (5-HT), and gamma-aminobutyric acid (GABA), play an important role in the development and regulation of pain. EAE has been shown to decrease levels of all three of these neurotransmitters in the spinal cord (Musgrave et al., 2011; Musgrave, Tenorio, Rauw, Baker & Kerr, 2011). These findings coincide with decreased levels of these neurotransmitters in MS, and could possibly be related to the disease progression and enhanced nociceptive sensitivity seen in EAE. EAE provides a starting point for researchers to examine the mechanisms of MS and the associated neuropathic pain.

1.3 Antidepressants in Pain

The first line of treatment for neuropathic pain are antidepressants, predominantly tricyclic antidepressants (TCA's) and selective norepinephrine-serotonin reuptake inhibitors

(SNRI's), although occasionally selective serotonin reuptake inhibitors (SSRI's) are prescribed (Ardid et al., 2001; Attal et al., 2010; Dworkin et al., 2007; Mika, Zychowska, Makuch, Rojewska & Przewlocka, 2013). TCA's, such as amitriptyline and desipramine, modulate noradrenergic and/or serotonergic function (both in the case of amitriptyline and primarily NA in the case of desipramine) by inhibiting reuptake of NA and 5-HT into nerve terminals (Mika et al., 2013; Sindrup, Otto, Finnerup & Jensen, 2005). These drugs produce a number of unpleasant side effects such as dry mouth, blurred vision, and weight gain, and overdosing with them can result in cardiotoxicity (Mika et al., 2013; Sindrup et al., 2005; Smith, 1998). SNRI's, such as venlafaxine, are more frequently prescribed for the treatment of neuropathic pain than SSRI's due to their superior analgesic effects over SSRI's (Mika et al., 2013; Smith, 1998). The difference in efficacy between SNRI's and SSRI's suggests that the noradrenergic system plays a significant role in pain (Gendreau et al., 2005; Marks et al., 2009; Stahl, Grady, Moret & Briley, 2005). Another class of antidepressants, the monoamine oxidase inhibitors (MAOI's), are used when other avenues such as SNRIs and SSRIs have failed. The MAOIs are a class of antidepressants that inhibit monoamine oxidases thus, preventing the breakdown of monoamines and leading to increased levels of NA and 5-HT in the CNS (Dworkin et al., 2007). Phenelzine is unique in that it also increases brain levels of GABA (Sowa, Holt, Todd & Baker, 2004).

It has been suggested that the therapeutic effect of antidepressants comes from their ability to increase levels of NA and 5-HT in the synaptic cleft, but increased GABA has been implicated in the efficacy of antidepressants as well (Attal et al., 2010; Benson et al., 2015; Mico´, Ardid, Berrocoso & Eschalier, 2006; Dupuis et al., 2016; Eide & Hole, 1993; Mika et al., 2013; Ortega, Fernández-Pastor, Callado & Meana, 2010). The relationship between chronic pain and levels of NA, 5-HT, and GABA is not fully understood, but all three individual

neurotransmitters, and their interactions, have been implicated in the development and regulation of pain. Further research must be conducted to determine why increasing levels of these neurotransmitters through antidepressant treatment benefits patients experiencing pain.

1.4 NA

NA is a prominent neurotransmitter that is synthesized from dopamine, and plays a role in the regulation of pain (Pertovaara, 2006; Pertovaara, 2013). The noradrenergic system spreads extensively throughout the nervous system, with the primary source for ascending and descending central nervous system (CNS) projections coming from the locus coeruleus (LC), as well as postganglionic nerve fibers in the periphery (Pertovaara, 2013). Ascending projections are implicated for the regulation of vigilance, attention, and other cognitive functions. This is mainly mediated via the dorsal and ventral bundles and periventricular bundle (Cooper, Bloom & Roth, 2003). Descending projections on the other hand are associated with the control of spinal motor output. The noradrenergic system also provides strong, primarily inhibitory, innervation to the spinal dorsal horns, an area that is essential in the pain pathway (Cooper et al., 2003; Pertovaara, 2013; Sawynok, 2003; Westlund & Coulter, 1980). An abundance of noradrenergic terminals in the spinal dorsal horn supports the idea that noradrenergic descending projections from the LC play an important role in regulating different aspects of somatomotor and autonomic function (Westlund & Coulter, 1980). This extensive innervation of the superficial dorsal horn by the noradrenergic system is proposed to be pain inhibitory, with the LC being considered a "pain suppressor" (Pertovaara, 2013). NA mediates its effects through its two catecholamine receptors, α- and β-adrenoceptors (Pertovaara, 2013; Ruffolo & Hieble, 1994). Subtypes of α- and β-adrenoceptors mediate different effects based on their location. α-Adrenoceptors (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}) are the primary modulators of pain and are located throughout the spinal

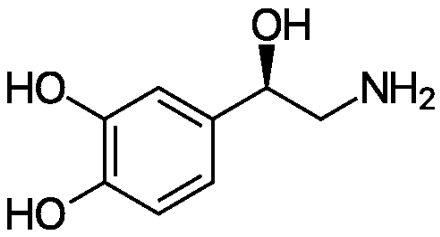

Figure 1. Molecular structure of noradrenaline (NA).

cord, though predominantly within the dorsal horn (Nalepa et al., 2005; Ruffolo & Hieble, 1994; Shi, Winzer-Serhan, Leslie & Hökfelt, 1999). α_2-Adrenoceptors are the main noradrenergic system mediators of pain suppression within the spinal dorsal horn (Ruffolo & Hieble, 1994; Yaksh & Malmberg, 1994).

1.5 5-HT

Another important neurotransmitter involved in pain processing and modulation is 5-HT, which is derived from tryptophan and produced in high levels in the raphe nuclei (Eide & Hole, 1993; Stamford, 1995). 5-HT plays a significant role in the regulation of pain and nociception within the CNS and is consistently observed to have antinociceptive effects (Dupuis et al., 2016; Eide & Hole, 1993; Sommer, 2004). The function 5-HT plays in pain is not fully understood, but it is suggested to contribute to inhibitory descending analgesia that projects to many areas, including regions of the spinal cord (Stamford, 1995). Yaksh and Wilson (1979)

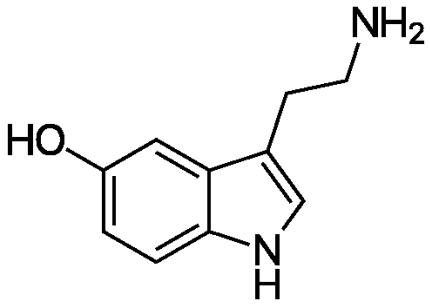

Figure 2. Molecular structure of serotonin (5-HT).

reported significant analgesic effects of 5-HT, which acted to decrease nociceptive behaviour in rats. 5-HT may also have effects outside the CNS, but in these peripheral locations it functions as an inflammatory pain mediator (Dray, 1995). The complexity of 5-HT's role is likely due to the variety of receptor subtypes within the CNS (Eide & Hole, 1993). 5-HT receptor subtypes include 5-$HT_{1,}$ 5-$HT_{2,}$ 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7, several of which play different roles in pain regulation, such as promoting inhibition within the dorsal horn (Eide & Hole, 1993; Liu, Su & Lin, 1988; Peng, Lin & Willis, 1996). Activation of the 5-HT_{2A} or 5-HT_{5A} receptor has been reported to mediate analgesic effects in pain conditions through inhibitory signalling cascades. Activation of these receptors provides relief from craniofacial and peripheral hyperalgesia (Dupuis et al., 2016; Hannon & Hoyer, 2008; Millan, 1995; Muñoz-Islas et al., 2014; Okamoto et al., 2007). In addition, the SSRI fluoxetine is proposed to provide pain relief via increased activity of 5-HT_{2A} receptors (Anjaneyulu & Chopra, 2004; Dupuis et al., 2016). Dupuis et al. (2016) also showed that dysfunctional 5-HT_{2A} receptors were implicated in neuropathic pain, supporting the idea of their involvement in the inhibitory actions of 5-HT in pain processing. Furthermore, 5-HT_{2A} receptor activation in conjunction with 5-HT_{1A} antagonism (acting to increase the onset of action) has been associated with the beneficial effects of antidepressants, which act to normalize inhibitory activity in the bulbospinal pathway in pain conditions (Ardid et al., 2001; Ardid et al., 1995; Dupuis et al., 2016; Mico´, Ardid, Berrocoso & Eschalier, 2006).

1.6 GABA

GABA is an abundant inhibitory neurotransmitter within the CNS and is synthesized from glutamate (GLU), and important excitatory neurotransmitter (Watanabe, Maemura, Kanbara, Tamayama & Hayasaki, 2002; Zeilhofer, Möhler & Lio, 2009). Synthesis of GABA

from GLU occurs via a decarboxylation reaction that is catalyzed by glutamate decarboxylase (GAD) (Watanabe et al., 2002). GABA is catabolized by GABA transaminase (GABA-T), which causes the degradation of GABA, and producing GLU as a bi-product, that is recycled for future GABA synthesis (Watanabe et al., 2002). GABA's role in pain production and modulation is not fully understood, but has been suggested to largely control the communication of nociceptive signaling from the periphery through the dorsal spinal cord and up to higher brain structures (Melzack & Wall, 1965). This function is thought to be through inhibitory GABAergic interneurons within the spinal dorsal horn, an important area implicated in pain (Melzack & Wall, 1965; Zeilhofer, 2008; Zeilhofer, Möhler & Lio, 2009). GABA serves to maintain inhibition within the CNS, therefore preventing innocuous stimuli from exciting neurons

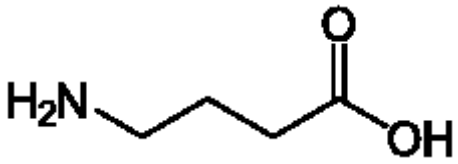

Figure 3. Molecular structure of gamma-aminobutyric acid (GABA)

associated with pain production (Zeilhofer, Möhler & Lio, 2009). $GABA_A$, $GABA_B$, and $GABA_C$ receptors mediate these effects, through a range of mechanisms (Chebib & Johnston, 1999). $GABA_A$ receptors are more widely distributed throughout the CNS and are thought to be the primary mediators of pain inhibition (Bormann, 2000; Bormann & Feigenspan, 1995; Jasmin, Wu & Ohara, 2004; Zeilhofer, Möhler & Lio, 2009). The blockade of $GABA_A$ receptors in the spinal cord produces severe pain hypersensitivity in animal models, and enhanced activity of $GABA_A$ receptors alleviates pain in both animals and humans (Enna & McCarson, 2006; Ugarte, Homanics, Firestone & Hammond, 2000; Yaksh, 1989; Zeilhofer, Möhler & Lio, 2009). Reduced levels of GABA within the spinal dorsal horn has been identified as a hallmark of pain, and effective treatments have been shown to increase GABA levels, resulting in analgesic effects

(Jasmin, Rabkin, Granato, Boudah & Ohara, 2003; Zeilhofer, Möhler & Lio, 2009). Interestingly, data have suggested that GABA is also released when 5-HT_{2A} receptors are activated, supporting the idea that both neurotransmitters play critical roles individually as well as having important interactions with one another to mediate analgesia (Dupuis et al., 2016). The role of GABA in pain processing and modulation is complex and remains to be fully elucidated, but overall its function seems to be one of inhibitory pain modulation (Jasmin et al., 2003).

Chronic pain is commonly treated with antidepressants that act to increase functional availability of NA, 5-HT, and GABA within the CNS (Dworkin et al., 2007). It is important to acknowledge that although each neurotransmitter plays their own individual roles in pain production and modulation, the interactions between them are also important to consider when studying the mechanisms underlying chronic pain.

1.7 Phenelzine (PLZ)

Phenelzine (PLZ) is a non-selective, irreversible MAOI that produces long-lasting increases in NA and 5-HT. Through the production of the active metabolite β-phenylethylidenehydrazine (PEH) it can elevate GABA levels via the inhibition of the enzyme

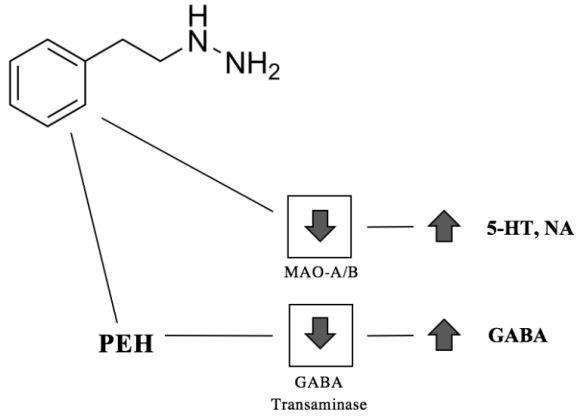

Figure 4. PLZ and its active metabolite PEH act to increase levels of NA, 5-HT, and GABA.

GABA-T (Mackenzie et al., 2010; Parent, Habib & Baker, 2000). PLZ is classified as a non-selective MAOI because it acts to inhibit both isoforms, MAO-A and MAO-B. MAO-A shows a higher affinity for NA, 5-HT, and Da, whereas MAO-B associates with PEA and deprenyl (Johnston, 1968; Knoll & Magyar, 1972; Shih & Thompson, 1999). PLZ treatment has been used for a variety of disorders, although primarily for depression and anxiety. Its anxiolytic effects can be observed clinically, as well as in animal models (Musgrave et al., 2011; Paslawski, Treit, Baker, George & Coutts, 1996). Female mice with EAE that were treated with PLZ were observed to have decreased anxiety and increased exploratory behavior in an 'open field' behavioural assay (Musgrave et al., 2011). In this paradigm, the movement of mice were tracked in an open field, and the number of times they crossed over the midline was recorded; increased crossings suggest decreased anxiety and increased exploratory behaviours. PLZ-treated mice showed significantly more crossings in the open field when compared to vehicle-treated EAE animals, suggesting that PLZ beneficially decreases anxiety-like behaviour, resulting in increased exploration (Musgrave et al., 2011). These effects are proposed to be a direct benefit of increased GABA levels, which is supported by the evidence that anxiety was not reduced in the elevated plus maze (EPM) when mice were treated with N^2-Acetylphenelzine (N2-Ac-PLZ), a derivative that has no elevating effect on GABA levels (Baker, Wong, Yeung & Coutts, 1991; Musgrave et al., 2011; Paslawski et al., 1996). Increases of NA, 5-HT, and GABA levels via PLZ administration have been demonstrated in both non-diseased, healthy animals as well as mice in with EAE (Matveychuk et al., 2013). In the EAE model, these increases have been accompanied by improved disease severity, delayed onset of the disease, and reduced mechanical hypersensitivity in female mice with EAE (Benson et al., 2013; Musgrave et al., 2011; Potter et al., 2016).

1.8 N^2-Acetylphenelzine (N2-Ac-PLZ)

N2-Ac-PLZ is an acetylated derivative of PLZ that is also a non-selective inhibitor of MAO's. N2-Ac-PLZ acts to increase NA and 5-HT levels through inhibition of monoamine oxidases, but dissimilar to PLZ, does not produce the active metabolite PEH, therefore does not increase levels of GABA (McKenna, Baker & Coutts, 1991; McKenna, Baker, Coutts & Greenshaw, 1992; Sowa et al., 2004). PLZ has been observed to have anxiolytic properties,

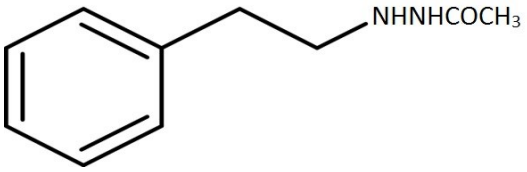

Figure 5. Molecular structure of N2-Ac-PLZ.

which were thought to be related to elevated GABA levels, and N2-Ac-PLZ has been reported to have no such effect (Musgrave et al., 2011). These disparities between PLZ and N2-Ac-PLZ provide the opportunity to study GABA's mechanisms and importance in the production and regulation of pain in EAE between the sexes.

1.9 Formalin: Effects of PLZ & N2-Ac-PLZ

The formalin test is a model of tonic pain processing that is commonly used to examine nociceptive behaviour (Dubuisson & Dennis, 1977). Mice receive a subcutaneous injection of formalin (37% formaldehyde solution in 0.9% saline) into the plantar surface of one hindpaw and are then observed for 30 to 60 minutes while the time spent engaging in nociceptive behaviours, such as licking, lifting, flinching of the injected paw, is recorded (Mifflin, Benson, Thorburn, Baker & Kerr, 2016). Mifflin et al. (2016) observed that treatment with PLZ or N2-Ac-PLZ prior to formalin significantly decreased nociceptive behaviours in male mice. The

response of female mice to PLZ, was however, less robust, and female mice were not responsive at all to pre-treatment with N2-Ac-PLZ (Mifflin et al., 2016). Interestingly the levels of NA and 5-HT were increased to a similar level in both female and male spinal cords following pre-treatment with N2-Ac-PLZ. These results suggest that MAO inhibitors, which act to increase levels of NA and 5-HT, can significantly benefit nociception in males within a tonic pain model but have mixed effects in females. Female mice were, however, responsive to pre-treatment with PEH to a similar level as males, indicating that females may require GABA to control nociception (Mifflin et al., 2016). The variance in nociceptive behavioural outcomes in comparison to similar spinal levels of neurotransmitters offers an interesting sex difference that should be explored further within a disease model with chronic pain.

1.10 Present Study: The Effects of N2-Ac-PLZ in the EAE Model of Multiple Sclerosis (MS)

The present study sought to investigate the potential therapeutic benefit of N2-Ac-PLZ treatment on disease parameters and nociceptive behaviours in EAE. I also sought to determine whether these effects were mediated by increased levels of NA and 5-HT in the CNS, and whether there are sex-differences in the response to treatment with N2-Ac-PLZ. The animal model EAE allowed us to ask several important questions regarding sex differences in EAE, as well as examine the effects of an antidepressant derivative in a non-clinical setting.

In experiment 1, I examined the effects of N2-Ac-PLZ on nociceptive, exploratory, and anxiety behaviours, as well as on the levels of NA, 5-HT, and GABA levels within the spinal cord in non-diseased, naïve mice of both sexes. These observations are important in order to understand the basal effects N2-Ac-PLZ in a non-diseased state. I predicted that treatment with N2-Ac-PLZ will increase levels of NA and 5-HT equally between female and male mice. Based

on previous results from the formalin assay, I predicted that N2-Ac-PLZ will be more effective at preventing pain hypersensitivity in naïve male mice.

In the next set of experiments, I assessed the effects of N2-Ac-PLZ on the disease course of EAE, as well as nociceptive, exploratory, and anxiety behaviours, and spinal cord neurotransmitter levels in both female and male mice with the disease. Based on results from the experiments conducted with PLZ and N2-Ac-PLZ in the formalin model, I predicted that N2-Ac-PLZ will increase NA and 5-HT levels equally in female and male mice with EAE, but only reduce pain hypersensitivity in males with EAE.

2. Methods

2.1 Subjects and Experimental Treatments. All mice (C57/BL6) were received from Charles River Canada Laboratory, Canada. Mice were group-housed (5 mice per cage) in a controlled room with a set 12-hour light/dark cycle throughout the entire experiment. All experimental procedures were conducted during the light phase. Mice were given free access to standard food pellets and water. All procedures were performed in compliance with the Canadian Council on Animal Care Guidelines and Policies with the approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta (00000274). The information below outlines the number of mice, age of mice, as well as procedures used for each experiment.

2.1-1 Naïve N2-Ac-PLZ Experiment

2.1-1.1 Female and male 6- to 8-week-old C57/BL6 mice were used for this experiment (n= 72; 36 females, 36 males). On the testing days, mice underwent morning baseline behavioural testing and then randomly divided into groups who received an intraperitoneal injection of a vehicle treatment (bacteriostatic water), N2-Ac-PLZ (39.82 mg/kg in bacteriostatic water; a dose calculated to be equivalent on a molar basis to PLZ treatment at 30 mg/kg), or no injection at all (naïve). Mice were then separately housed for three hours to allow for maximum effect of the injections (McManus, Baker, Martin, Greenshaw & McKenna, 1992), then run through the same behavioural tests (outlined below). N2-Ac-PLZ was obtained from the laboratory of Dr. Glen Baker (Department of Psychiatry, University of Alberta, Edmonton, Alberta, Canada).

2.1-1.2 Behavioural Testing. Prior to testing, all mice were left undisturbed for 2-3 days in their home cages after arrival from Charles River Canada. This was done to allow

the animals to acclimatize to their new environment. Mice were also handled by the experimenter for 3-5 days prior to baseline testing to ensure they were familiar with the experimenter conducting the behavioural tests. Baseline testing was conducted the week prior to any treatment. This two-week habituation period allowed the mice to properly acclimatize to their environment, as well as ensuring that mice reached sexual maturity, an important point to consider as potential sex differences were of interest in these studies. The behavioural tests used in each experiment are outlined below.

2.1-1.2.1 *Exploratory Behaviour Testing.* The open field test was used to assess exploratory behaviours. A subset of mice (n=36; 18 females, 18 males) were individually placed in an open field box and recorded to analyze behaviour. Software was used to determine how much the animal explored the open field, and how much time was spent in the center field (Ethovision version 9.0). Mice underwent one baseline open field test a week before treatment to minimize habituation, and then were tested once after drug treatment.

2.1-1.2.2 *Anxiety Testing.* The elevated plus maze (EPM) was used to measure anxiety behaviour. A subset of mice (n= 36; 18 females, 18 males) was individually placed in the center of a plus shaped apparatus, where two of the arms are closed, and two of the arms are open. Time spent in open arms, as well as number of entries into the open arms was measured, with increases in both indicating decreased anxiety. Mice were tested only after treatment.

2.1-1.2.3 *Mechanical Hypersensitivity Testing.* Von-Frey hairs were used to measure mechanical hypersensitivity, where the pain threshold was determined by the

lowest force (in grams) that caused a withdrawal response (i.e. shake, lift, or lick) in three of five repetitive stimuli on the base of their hindlimb paws; a lower threshold indicated increased hypersensitivity. A baseline test was conducted in the morning prior to treatment and an experimental test was done three hours after treatment for a subset of mice (n= 36; 18 females, 18 males).

2.1-1.2.4 Motor Function Testing. The rota-rod was used to measure gross locomotor ability. A subset of mice (n= 36; 18 females, 18 males) were individually placed on a rota-rod (a spinning rod) that was fixed at 12 rpm, and the duration they were able to stay on was measured for three trials (up to 180 s) and then averaged. The longer the mouse stays on the rod, the better the motor function. A baseline test was conducted in the morning prior to treatment and an experimental test was done three hours after treatment.

2.1-1.3 Tissue Collection Procedure. At the end of testing, all mice were euthanized via a Euthansol (sodium phentobarbital) intraperitoneal injection and then transcardially perfused with 0.9% saline. Whole spinal cord tissue samples were collected and flash frozen in liquid nitrogen. Tissue was stored at -80°C prior to analysis.

2.1-1.4 High Performance Liquid Chromatography (HPLC). Samples were retrieved from the -80°C freezer and kept on dry ice to prevent thawing, then weighed from all experiments. Tissue was prepared for HPLC analysis to determine levels of amino acids and biogenic amines following a protocol similar to Musgrave et al. (2011). *Amino Acids.* To prepare samples for amino acids, the tissue was homogenized in 5 volumes of ice-cold water. An aliquot was added to 4 volumes of ice-cold methanol and left on ice for 10 minutes, then centrifuged (12,000 *g* for 4 minutes). The

supernatant was diluted with distilled water to a final 120-fold dilution. Part of the final supernatant was reacted with o-pthaldialdehyde and N-iso-butyryl-L-cysteine dissolved in a borate buffer and 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 run of samples.

Biogenic Amines. To prepare samples for measurement of biogenic amines, aliquots of tissues homogenized in 5 volumes of water were combined with $1/10^{th}$ the volume of ice-cold 1 M $HClO_4$ containing ascorbic acid (500 μm) and ethylenediaminetetraacetic acid (100%/mg). Homogenates were vortexed and centrifuged at 12,000*g* for 4 minutes. Subsequent supernatants were used for HPLC analysis using electrochemical detection. Applied potential for electrochemical detection was 0.65 V, and calibration curves were constructed for each point. All water was distilled and purified by reverse osmosis using the Milli-Q filtration system from Millipore (Billerica, Massachusetts). Methanol, tetrahydrofuran, and acetonitrile were HPLC-grade obtained from Fisher Scientific (Pittsburgh, PA). 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.

2.1-1.5 Statistical Analyses. Analyses were conducted using Sigmaplot software version 12.0 (Systat Software, Inc., San Jose, CA) using t-tests, one way ANOVAs, one way repeated measure ANOVAS, or three way ANVOAs. Post-hoc analyses were

conducted if needed. If data failed to meet normal distribution criteria, nonparametric tests were used where applicable. For all tests, significance was set at $P < 0.05$.

2.1-2 ***Disease Related Chronic Pain Experiment.***

2.1-2.1 Female and male 6- to 8-week-old C57/BL6 mice were used for this experiment (n= 80; 40 females, 40 males).

EAE Induction. Mice in this experiment were given EAE to induce disease-related chronic pain. It has previously been shown that this animal model of multiple sclerosis is associated with chronic pain (Aharoni, 2013; Olechowski et al., 2009). EAE is induced using myelin oligodendrocytes glycoprotein 35-55 (MOG_{35-55}) (Peptide Synthesis Facility, University of Calgary, Alberta, Canada). Forty-four mice were induced with a 50 μg subcutaneous injection of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA) at a concentration of 1.5 mg/ml. Thirty-six mice served as controls (CFA mice), and only received subcutaneous injections of CFA. Both EAE and CFA mice received an intraperitoneal injection of 300 ng of pertussis toxin (List Biological laboratories, Cedarlane, Canada) on the day of induction and 48 h later. Mice were monitored daily (34 days post EAE induction) for clinical signs of EAE and graded according to the following scale: Grade 0, normal mouse; Grade 1, flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both. This clinical scoring was used to track the disease course of the animals, as well as the effectiveness of treatment with N2-Ac-PLZ. To examine the effectiveness of N2-Ac-PLZ in reducing disease-

related chronic pain, EAE and CFA mice were divided randomly into drug treatment groups. Mice received either an injection of vehicle treatment (bacteriostatic water) or N2-Ac-PLZ (39.82 mg/kg in bacteriostatic water) every other day. Injections began seven days post EAE induction.

2.1-2.2 Behavioural Testing. All testing followed the same methods as presented in **Naïve N2-Ac-PLZ experiment** (*2.1-1*).

2.1-2.2.1 Exploratory Behaviour Testing. A subset of mice (n= 40; 20 females, 20 males) underwent three days of baseline testing, testing at onset of clinical signs, and once per week for the duration of the experiment. Mice underwent multiple testing time points in this experiment, as EAE and sickness in general is known to reduce exploratory behaviour.

2.1-2.2.2 Mechanical Hypersensitivity Testing. Mice underwent three days of baseline testing, testing at onset of clinical signs, and once per week for the duration of the experiment.

2.1-2.2.3 Motor Function Testing. Mice underwent baseline testing, testing at onset, and testing once per week.

2.1-2.3 Tissue Collection Procedure, *HPLC*, and *Statistical Analyses.* All followed the same procedures as outlined in **Naïve N2-Ac-PLZ experiment** (*2.1-1.3*, *2.1-1.4*, *2.1-1.5*).

3. Results

3.1 Effects of N2-Ac-PLZ in Naïve Mice

To explore the baseline effects of N2-Ac-PLZ, I examined its effects after acute administration in a variety of behavioural assays, as well as its influence on neurotransmitter levels in naïve mice of both sexes.

3.1-1 Exploratory Behavior in the Open Field Test. I used the open field test to examine exploratory behaviour in naïve female and male mice who were pretreated with vehicle, N2-Ac-PLZ, or no injection at all. After the IP injection, mice were individually housed for 3 hours to allow for maximum effect of the treatment. The overall total distance moved around the open field and time spent in the center zone of the field were analyzed for each treatment group, and compared to a baseline level for both measures. Increased distance moved and time spent in the center zone suggests that exploratory behavior is increased in these animals. Our lab previously reported that PLZ increased the number of line crossings within the open field test, indicating that exploratory behavior was increased, but suggested that these effects were due to the increased GABA levels (Musgrave et al., 2011). I tested this theory by treating naïve mice with N2-Ac-PLZ, which lacks the active metabolite PEH, therefore not increasing GABA levels.

There was a significant effect in total distance moved in females (one-way ANOVA: $F_{3, 35} = 6.119$, P = 0.002; Fig 6A), with a significant decrease in distance moved from baseline to no treatment (NT) and vehicle treated mice (Tukey's post hoc, NT: q = 5.208, p = 0.005; Veh: q = 4.300, p = 0.023). An effect was also found for total time spent in the center zone in female mice (one-way ANOVA, $H_3 = 10.457$, P = 0.015; Fig 6B). Non-treated female mice were observed to spend significantly less time in the center when compared to baseline

(Dunn's post hoc, Q = 2.778, p = 0.033). These results indicate that in general, exploratory behaviour is decreased when compared to the baseline. These results may be due to any stress the animals experience on the day of the experiment, including transport and being housed individually after treatment, as well as habituation to the behavioural test and once novel environment.

The exact same test was performed on males to observe any potential sex differences in behaviour with N2-Ac-PLZ treatment. There was a significant effect in total distance moved in male mice (one-way ANOVA, $H_3 = 12.945$, P = 0.005; Fig 6C). Male mice treated with vehicle were observed to move significantly less when compared to baseline levels (Dunn's post hoc, Q = 3.445, p = 0.003). A significant effect was also found for male time spent in the center zone (one-way ANOVA, $H_3 = 12.620$, P = 0.006; Fig 6D), where male NT mice spent significantly less time in the center zone of the open field (Dunn's post hoc, Q = 3.220, p = 0.008). In general, males showed decreased total distance moved and time spent in the center zone.

N2-Ac-PLZ treatment in females and males did not significantly increase total distance moved or time spent in the center zone, indicating that it has no significant effects on exploratory behaviour. These results support the hypothesis that Musgrave et al. (2011) proposed, and suggest that improved exploratory behaviour may be regulated through a GABAergic system.

3.1-2 Anxiety-like Behaviour in the Elevated Plus Maze. To determine if N2-Ac-PLZ could beneficially reduce anxiety, I used the elevated plus maze (EPM) to evaluate any baseline anxiolytic effects in naïve female and male mice. I used the number of entries into the open arms, as well as time spent in the open arms, as outcome measures, with increased entries

and time indicating reduced anxiety. Analysis of the number of entries into the open arms of both female and male mice as well as the time spent in open arms, did not reveal any significant differences between non-treated control mice and those that were treated with vehicle or N2-Ac-PLZ (Fig 7A-D). However there seems to be a trend towards normalization of time spent in open arms for both sexes treated with N2-Ac-PLZ (Fig 7B,D).

3.1-3 Mechanical sensitivity in the Von-Frey Hair Test. To examine the effects of N2-Ac-PLZ on baseline nociceptive sensitivity, I used the Von-Frey hair test in order to measure responsiveness to mechanical stimuli. Stimulating the plantar surface of the hind paws, I used stimuli with varying amounts of bending force in ascending order to elicit a response (i.e. shake, lift, lick). The Von Frey filament that produced responses in at least three out of five stimuli was deemed the 'threshold'. I did not observe any changes in withdrawal thresholds when compared to baseline levels in either sex (Fig 8A,B).

3.1-4 Motor Functioning in the Rota-Rod Test. The rota-rod test was used to determine whether N2-Ac-PLZ had any effect on locomotor function. Analysis did not reveal any significant differences in female or male mice between baseline and treatments (Fig 9A,B). Both female and male mice remained on the rota-rod for the full duration of tests.

3.1-5 Levels of Neurotransmitters in the Spinal Cord. HPLC was used to assess the levels of NA, 5-HT, and GABA within post-mortem spinal cord tissue. N2-Ac-PLZ has been reported to increase NA and 5-HT levels due to its inhibitory effects on MAO's, but does not affect GABA levels (McKenna et al., 1991; McKenna et al., 1992; Sowa et al., 2004). All graphs are expressed as a percent change from baseline. As expected, N2-Ac-PLZ treatment significantly increased the levels of NA and 5-HT in female mice (t-test, $U = 6.000$, $P<0.001$, Fig 10A; $U = 0.000$, $P<0.001$, Fig 10B) and in male mice (t-test, $t(21) = -4.129$, $P = 0.0005$;

Fig 10D; U = 4.000, P<0.001, Fig 10E). Changes in the levels of NA and 5-HT were notably greater in female mice compared to males [NA: 113.25% (female) vs. 63.07% (male); 5-HT: 455.44% (female) vs. 214.60% (male)]. N2-Ac-PLZ had no effect on GABA levels in either female or male mice (Fig 10C,F). These results provide evidence that N2-Ac-PLZ is increasing levels of NA and 5-HT, but not affecting GABA levels.

3.2 Effects of N2-Ac-PLZ in Mice with EAE

To explore the potential beneficial effects of N2-Ac-PLZ in a chronic disease paradigm, I administered N2-Ac-PLZ chronically to mice with EAE of both sexes and examined its effects on disease parameters, behavioural assays, and neurotransmitter levels. CFA control mice or mice with EAE were randomly sorted into treatment conditions, and either received vehicle or N2-Ac-PLZ treatment every other day.

3.2-1 Disease Course and Day to Onset. Animals were monitored daily to assess their clinical score according to the following scale: Grade 0, normal mouse; Grade 1, flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both. This was done to monitor their clinical progression throughout the experiment and record their day of disease onset (the day when they first present as clinical grade 1). Analysis did not reveal any significant differences in female or male clinical progression (Fig 11A,C), or day to onset with N2-Ac-PLZ treatment (Fig 11B,D), although the data indicate a trend towards delayed onset for females (Fig 11B). In addition, it should be noted that male mice had a significantly higher average peak clinical score than females, suggesting that disease severity is greater in male mice than in females (t-test, U = 120.000, P = 0.018; Table 1).

3.2-2 Exploratory Behavior in the Open Field Test. Exploratory behavior was measured by the total distance moved by female and male mice with EAE at onset (clinical grade 1), as well as time spent in the center zone of the open field, and compared to baseline levels. There was a significant reduction in the total distance moved in female mice with EAE (one-way ANOVA, $F_{2, 19} = 9.852$, $P = 0.001$; Fig 12A). Total distance moved significantly decreased in females treated with vehicle as well as N2-Ac-PLZ (Tukey post hoc, $q = 4.637$, $p = 0.012$; $q = 5.535$, $p = 0.003$). In addition, N2-Ac-PLZ did not have any significant effects on the amount of time spent in the center zone (Fig 12B).

Similarly, there was a significant reduction in the total distance moved by male mice with EAE (one-way ANOVA, $F_{2, 19} = 111.554$, $P < 0.001$; Fig 12C). Distance traveled in the open field was significantly decreased in male mice with EAE receiving either vehicle or N2-Ac-PLZ when compared to baseline levels (Tukey post hoc, $q = 17.083$, $p < 0.001$; $q = 17.409$, $p < 0.001$). Unlike the female mice with EAE, there was a significant effect in the amount of time that males spent in the center zone (one-way repeated measures ANOVA, $F_{2, 19} = 28.195$, $P < 0.001$; Fig 12D). Male mice with EAE who were treated with a vehicle or N2-Ac-PLZ spent significantly less time in the center zone when compared to baseline (Bonferroni post hoc, $t = 6.949$, $p < 0.001$; $t = 5.940$, $p = 0.001$).

In general, female and male mice with EAE moved significantly less, regardless of treatment condition. The time spent in the center zone also decreased in females and males, although this only reached statistical significance in male mice. These effects may be due to habituation, stress, or increased nociceptive sensitivity the mice experienced during the experiment (i.e. transport, housing, disease course).

3.2-3 Mechanical Hypersensitivity in the Von-Frey Hair Test. The Von-Frey hair task was used to measure mechanical sensitivity in female and male CFA treated control mice and mice with EAE. Withdrawal thresholds were determined by the force (grams) that elicited a response (i.e. shake, lift, lick) in three of five mechanical stimuli. All graphs are expressed as a percent change from baseline. N2-Ac-PLZ treatment significantly increased withdrawal thresholds in female mice with EAE (t-test, t (13) = -2.355, P = 0.035; Fig 13B). Withdrawal thresholds were normalized with N2-Ac-PLZ treatment compared to EAE mice treated with vehicle. This decrease in withdrawal thresholds in vehicle-treated females suggests that these EAE mice experienced allodynia (when a previously innocuous stimulus now elicits a painful response), which has been previously reported (Olechowski et al., 2009). N2-Ac-PLZ treatment did not have an effect on withdrawal thresholds in female CFA control mice (although it should be noted that the data suggest a trend towards an increase in thresholds when treated with N2-Ac-PLZ; Fig 13A). There were no significant differences in the withdrawal thresholds of either male CFA control mice (Fig 13C) or male mice with EAE that were treated with N2-Ac-PLZ (Fig 13D).

3.2-4 Motor Functioning in the Rota-Rod Test. The rota-rod test determines the locomotor function of the mice, which is important when dealing with mice with EAE since their motor abilities are affected later on in the disease course. However, motor function was not significantly changed when assessed at disease onset (the time in which Von Frey hair testing took place) and N2-Ac-PLZ treatment did not appear to have any effect on motor behaviour in male mice from both the CFA or EAE groups (Fig 14A-D).

3.2-5 Levels of Neurotransmitters in the Spinal Cord. HPLC was used to measure neurotransmitter levels in post-mortem spinal cord tissue. All graphs are expressed as a

percent difference from CFA vehicle controls. Previous data in naïve animals showed that N2-Ac-PLZ increased NA and 5-HT levels in both female and male mice. I performed the same HPLC analysis in EAE tissue to confirm that N2-Ac-PLZ increases these neurotransmitters in a disease-related chronic pain paradigm.

A sex and drug interaction was found for NA levels (three-way ANOVA, $F_{1, 57} = 4.258$, $P = 0.044$; Fig 15A,D). N2-Ac-PLZ administration significantly increased NA levels in female and male mice with CFA and EAE (Holm-Sidak post hoc, female: $t = 6.789$, $p < 0.001$, Fig 15B; male: $t = 3.398$, $p = 0.001$, Fig 15E). In general, females treated with N2-Ac-PLZ had a larger increase of NA levels when compared to males (Holm-Sidak, $t = 2.817$, $p = 0.007$).

An interaction between group and drug was found for 5-HT levels in male and female mice as well (three-way ANOVA, $F_{1, 50} = 25.035$, $P < 0.001$; Fig 15B,E). Treatment with N2-Ac-PLZ acted to significantly increase 5-HT levels in both female and male mice with CFA or EAE (Holm-Sidak post hoc, $t = 10.100$, $p <0.001$; $t = 2.418$, $p = 0.020$).

Surprisingly, a sex and drug interaction was found for GABA levels (three-way ANOVA, $F_{1, 55} = 4.499$, $P = 0.039$; Fig 15C,F). Administration of N2-Ac-PLZ significantly decreased GABA levels in female and CFA mice (Holm-Sidak post hoc, $t = 2.492$, $p = 0.016$). Additionally, GABA levels decreased in both female and male mice treated with a vehicle (Holm-Sidak post hoc, $t = 5.348$, $p < 0.001$).

3.3 Figures

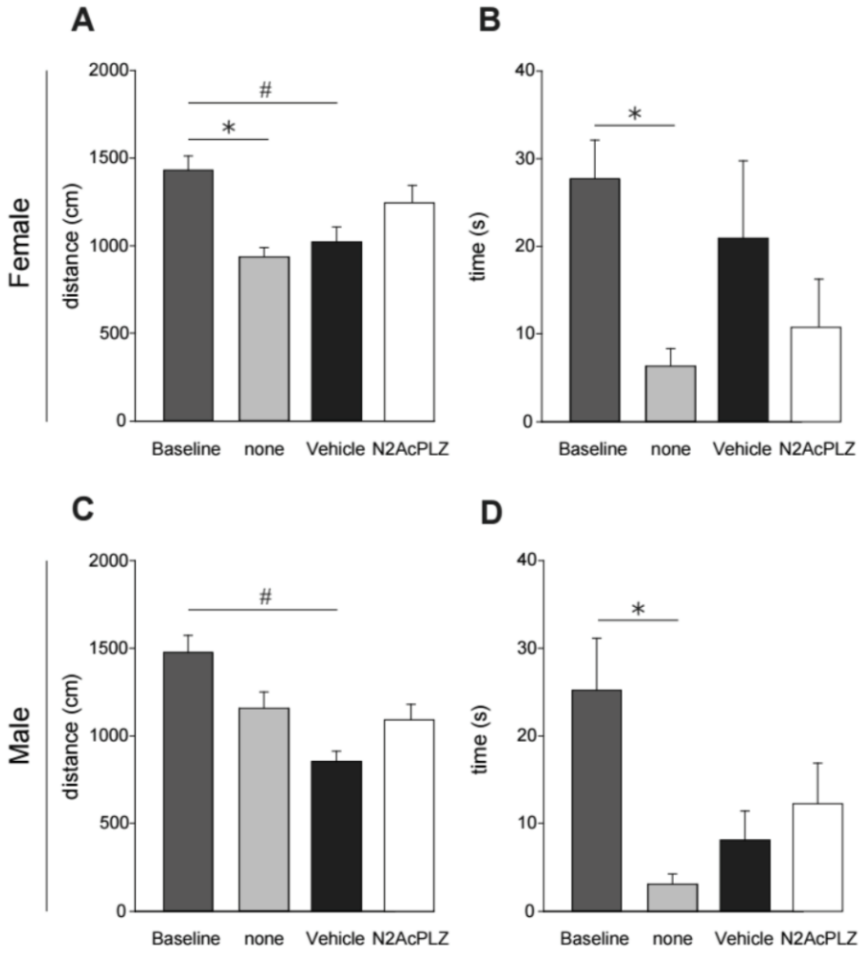

Figure 6. Exploratory behaviour measured using the open field test in naïve mice. Baseline (dark gray bars) represents testing that was done a week before the treatments. None (light gray bars) were the naïve mice that received no treatment (NT), vehicle (black bars) were the mice that received a vehicle injection (bacteriostatic water), and N2-Ac-PLZ (white bars) were the mice that received the injection of N2-Ac-PLZ on the experimental day. A) Total distance moved in female mice. Non-treated and vehicle-treated female mice were observed to move significantly less when compared to baseline levels. There was no effect on total distance moved in female mice treated with N2-Ac-PLZ. B) Female time spent in the center zone of the open field. Non-treated females spent significantly less time in the center zone compared to baseline levels. Treatment with a vehicle or N2-Ac-PLZ had no effect. C) Total distance moved in male naïve mice. Male mice who received the vehicle moved around significantly less when compared to baseline levels. N2-Ac-PLZ had no significant effect on distance moved. D) Male time spent in the center zone of the open field. Non-treated male mice spent significantly less time in the center zone compared to baseline. $*,^{\#}p<0.05$, One-Way ANOVA followed by *post hoc* Tukey or Dunn's analysis. Data are means ± standard error of the mean (SEM).

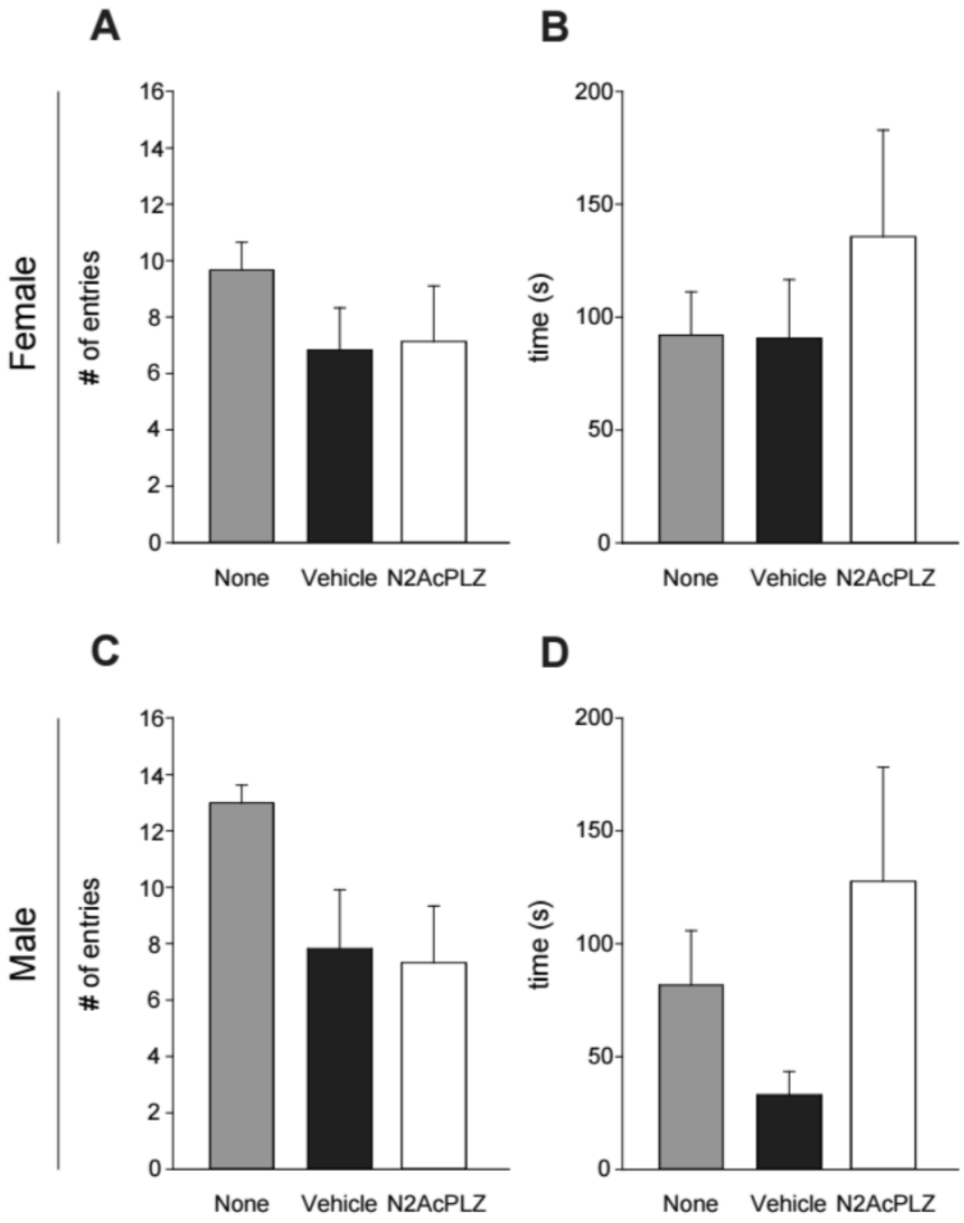

Figure 7. Anxiety behaviour measured in naïve mice using the elevated plus maze (EPM). None (light gray bars) represents the naïve mice that did not receive any treatment (NT), vehicle (black bars) were the mice that received a vehicle injection, and N2-Ac-PLZ (white bars) were the mice that received the N2-Ac-PLZ injection. The more entries and time spent in the open arms indicates that the animals were less anxious. A, C) N2-Ac-PLZ treatment did not significantly affect the number of entries into the open arms for females or males. B, D) N2-Ac-PLZ treatment did not significantly increase the amount of time spent in the open arms in females or males. Data are means ± SEM.

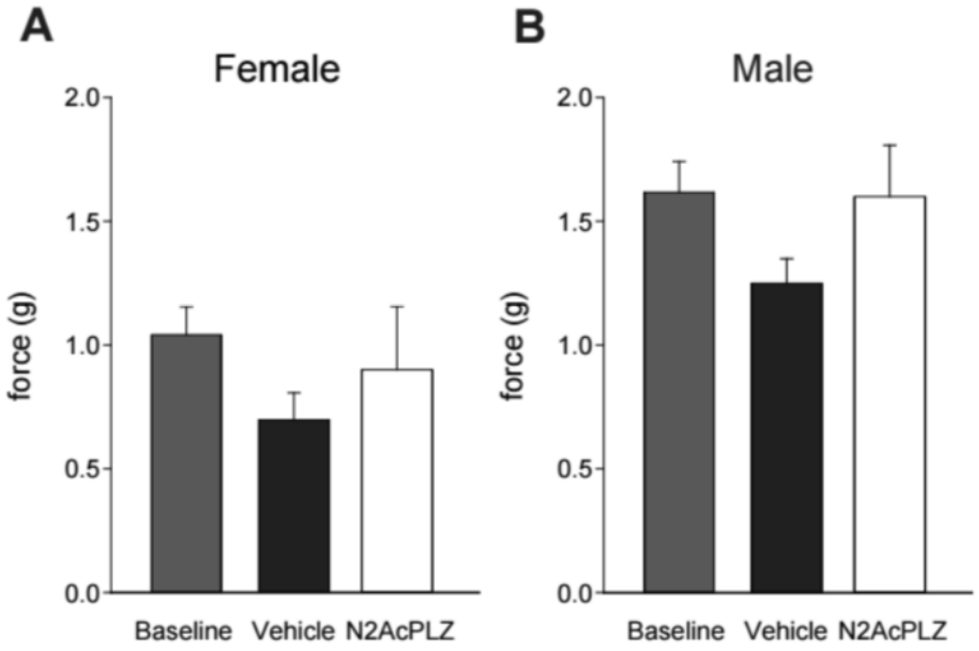

Figure 8. Von-Frey hairs were used to assess mechanical sensitivity in naïve mice. Baseline (dark gray bars) represents the responses that were recorded the morning of testing, vehicle (black bars) were the mice that received a vehicle injection, and N2-Ac-PLZ (white bars) were the mice that received the N2-Ac-PLZ injection. A, B) Treatment did not produce any significant changes in response thresholds in either females or males. Data are means ± SEM.

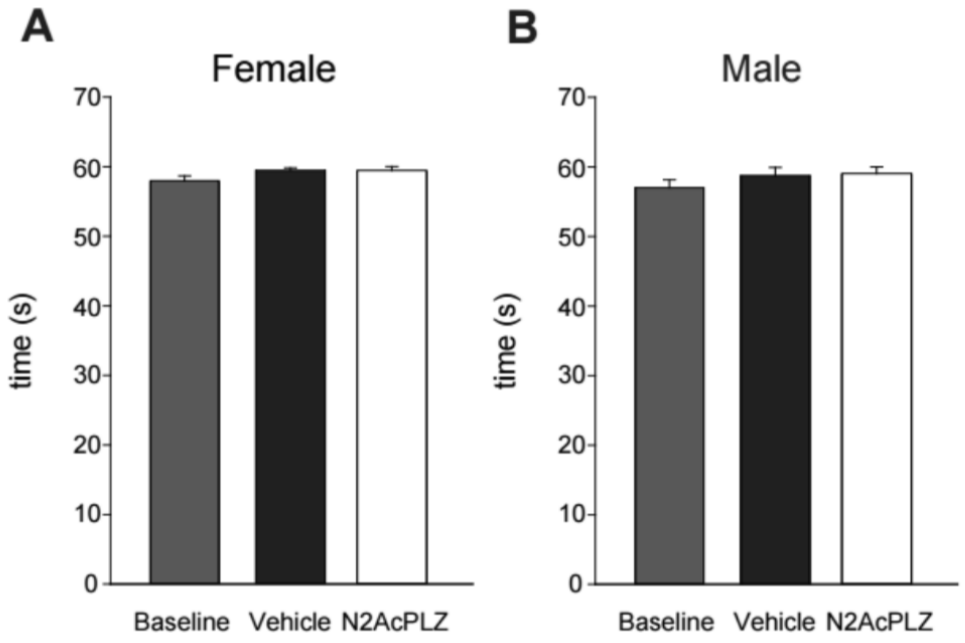

Figure 9. Naïve mice were tested on the rota-rod as a locomotor control measure. This ensures that any differences in behaviour were due to treatment and not locomotor ability. Baseline (dark gray bars) represents the responses that were recorded the morning of testing, vehicle (black bars) were the mice that received a vehicle injection, and N2-Ac-PLZ (white bars) were the mice that received the N2-Ac-PLZ injection. A, B) Treatment with a vehicle or N2-Ac-PLZ did not impair motor function in females or males. Data are means ± SEM.

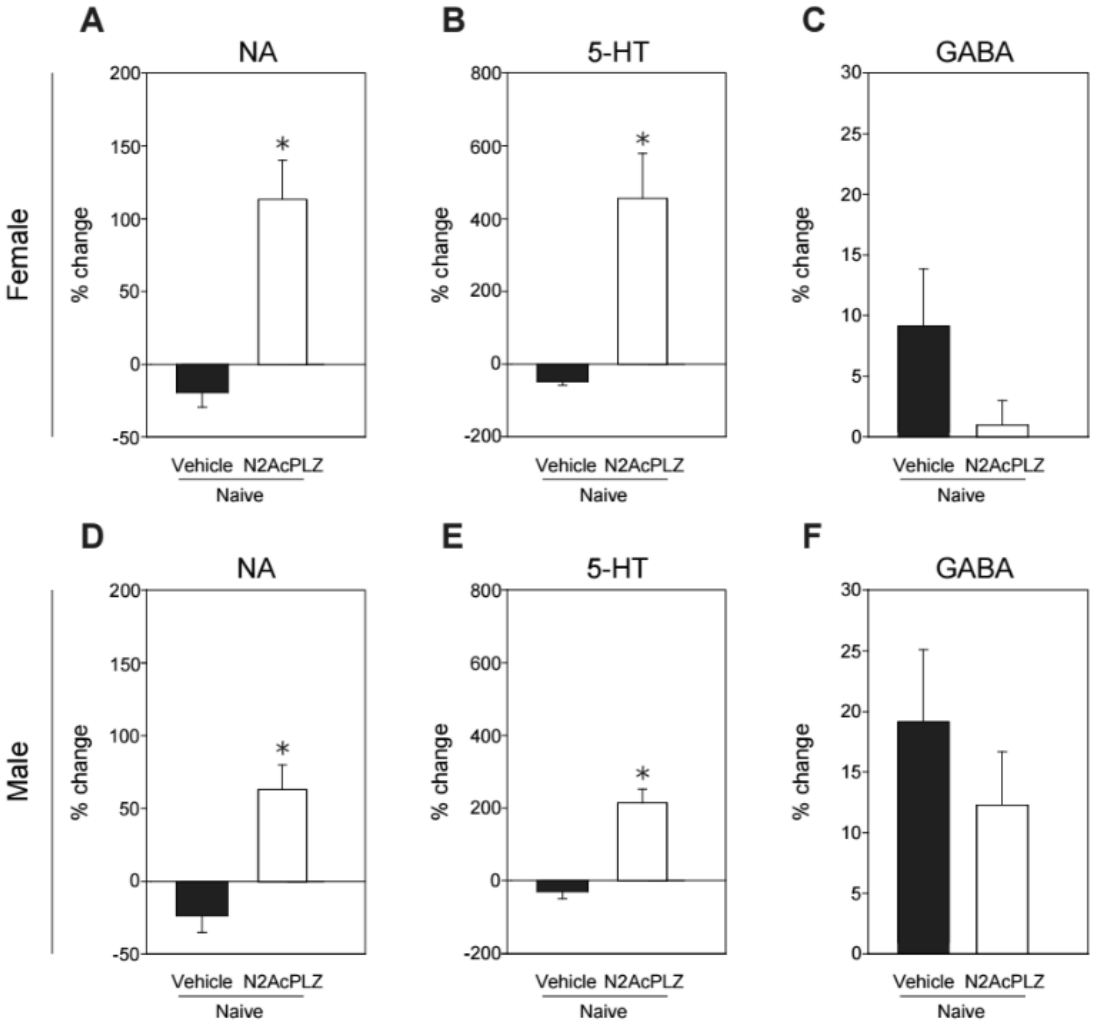

Figure 10. Spinal levels of NA, 5-HT, and GABA were measured using high performance liquid chromatography (HPLC) as compared to naïve (no treatment) controls. Black bars represent naïve mice treated with a vehicle and white bars represent naïve mice treated with N2-Ac-PLZ. All bars represent percent difference from naïve. A, D) Treatment with N2-Ac-PLZ significantly increased female and male NA levels. B, E) Treatment with N2-Ac-PLZ significantly increased female and male 5-HT levels. The effect of N2-Ac-PLZ on NA and 5-HT is greater in females than males. C, F) Treatment with N2-Ac-PLZ did not significantly change female or male GABA levels. $*,^{\#}p<0.05$, t-test. Data are means ± SEM.

Table 1. Peak clinical scores that female and male mice with EAE reached, with the number 5 representing a mouse that died. *$p<0.05$, t-test.

	Peak Scores	
	Female	**Male**
	5	4
	4	4
	4	4
	4	4
	4	4
	1	4
	4	4
	3	3
	4	3
	4	4
	3	4
	1	5
	4	4
	2	5
	3	5
	3	5
	4	5
	4	4
		5
		3
		4
		4
Average	3.39	4.14

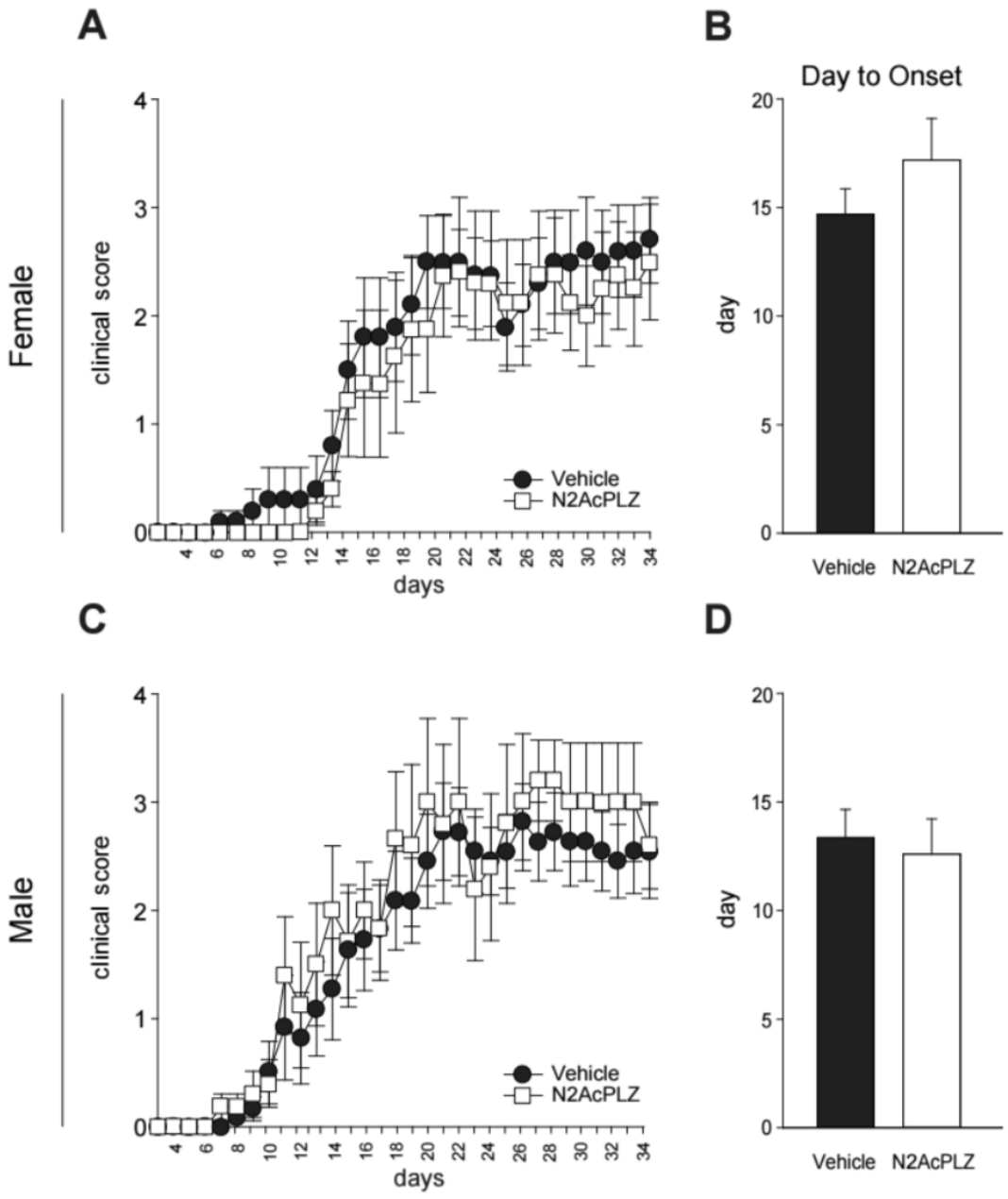

Figure 11. The effects of N2-Ac-PLZ on clinical scores of mice with EAE. Vehicle (black dots and bars) represents the mice with EAE that were treated with vehicle injections every other day, and N2-Ac-PLZ (white boxes and bars) were the mice with EAE that were treated with N2-Ac-PLZ injections every other day. A, C) There was no significant difference between female or male mice treated with vehicle or N2-Ac-PLZ. B, D) Day to onset was not significantly different between female or male mice treated with either vehicle or N2-Ac-PLZ. Data are means ± SEM.

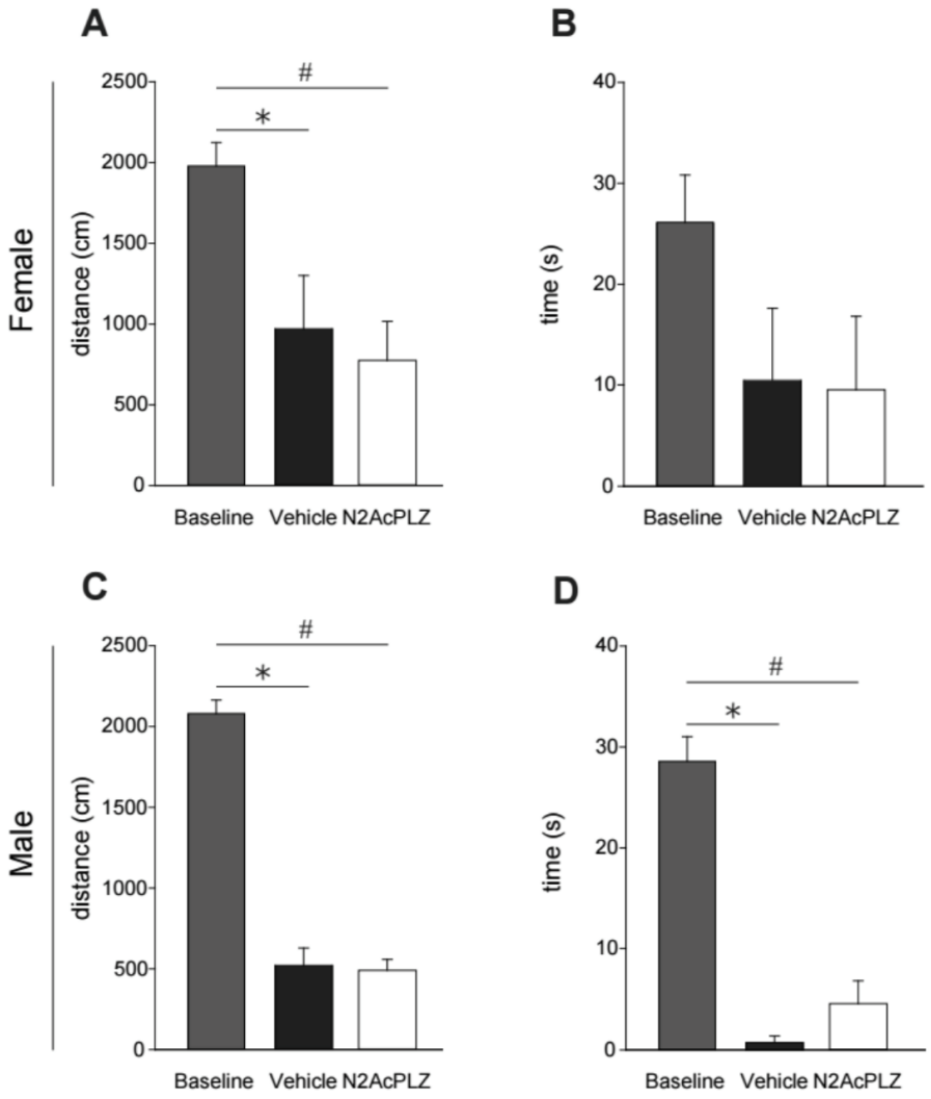

Figure 12. Exploratory behaviour in mice with EAE measured using the open field test. Baseline (dark gray bars) represents testing that was done before EAE induction, none (light gray bars) were the naïve mice that received no treatment (NT), vehicle (black bars) were the mice that received a vehicle injection (bacteriostatic water), and N2-Ac-PLZ (white bars) were the mice that received the injection of N2-Ac-PLZ. A) Total distance moved in female mice with EAE. Treatment with vehicle or N2-Ac-PLZ significantly decreased the total distance moved in the open field test when compared to baseline. B) Time female mice with EAE spent in the center zone of the open field. Treatment did not significantly affect females' time spent in the center zone. C) Total distance male mice with EAE moved. Vehicle or N2-Ac-PLZ treatment significantly decreased the total distance moved when compared to baseline. D) Time male mice with EAE spent in the center zone. Mice treated with vehicle or N2-Ac-PLZ spent significantly less time in the center zone when compared to baseline times. $*,^{\#}p<0.05$, One-Way ANOVA or One-Way Repeated Measures ANOVA followed by *post hoc* Tukey or Bonferroni analysis. Data are means ± SEM.

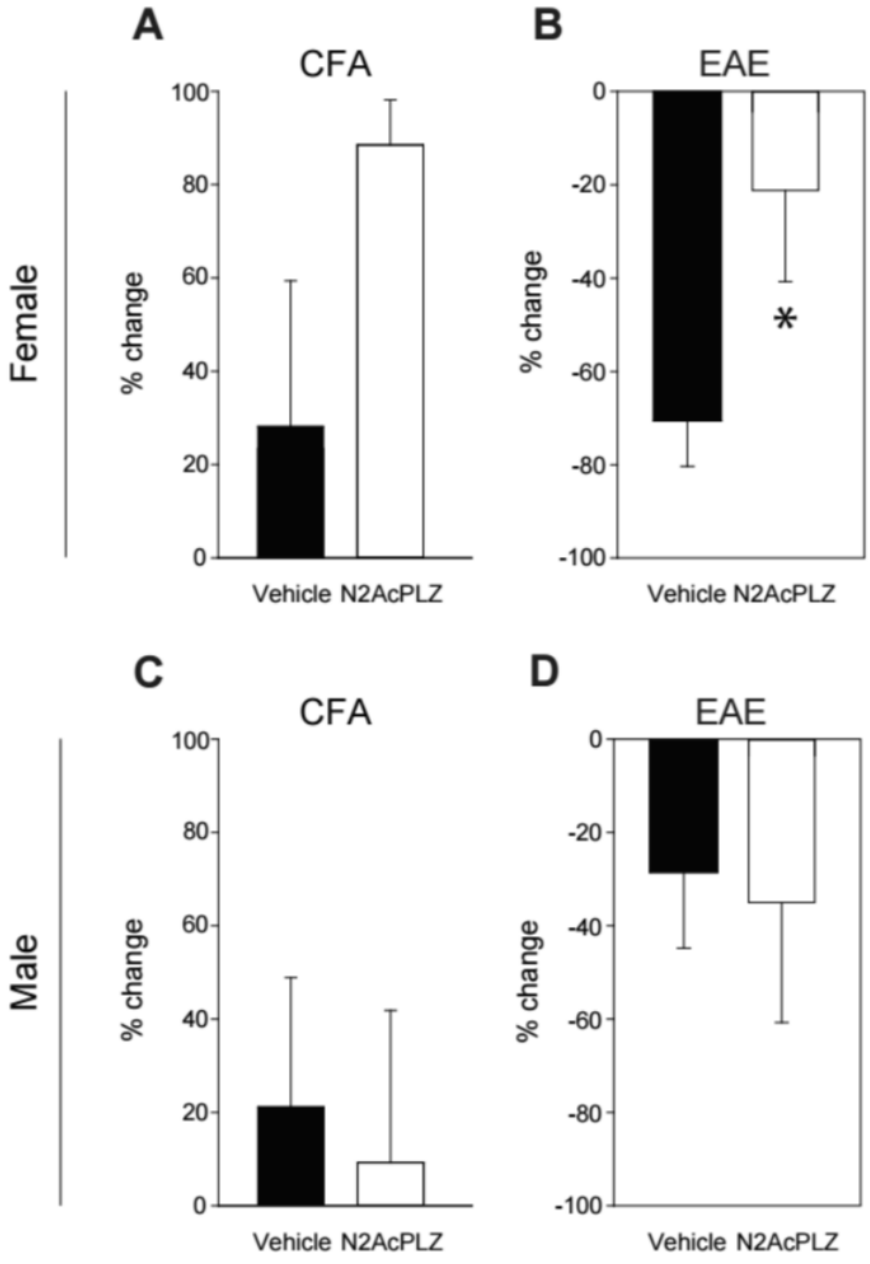

Figure 13. Von-Frey hairs were used to measure mechanical sensitivity in mice with EAE. CFA and EAE mice received either vehicle (black bars) or N2-Ac-PLZ (white bars) treatment every other day. All bars represent percent difference from naïve (CFA mice treated with vehicle). A, C, D) Withdrawal thresholds in the Von-Frey test. N2-Ac-PLZ administration does not significantly affect female CFA mice, or male CFA or EAE mice. B) Withdrawal thresholds to Von Frey hair mechanical stimulation are significantly increased in female EAE mice after N2-Ac-PLZ treatment when compared to vehicle-treated mice. $*,^{\#}p<0.05$, t-test. Data are means ± SEM.

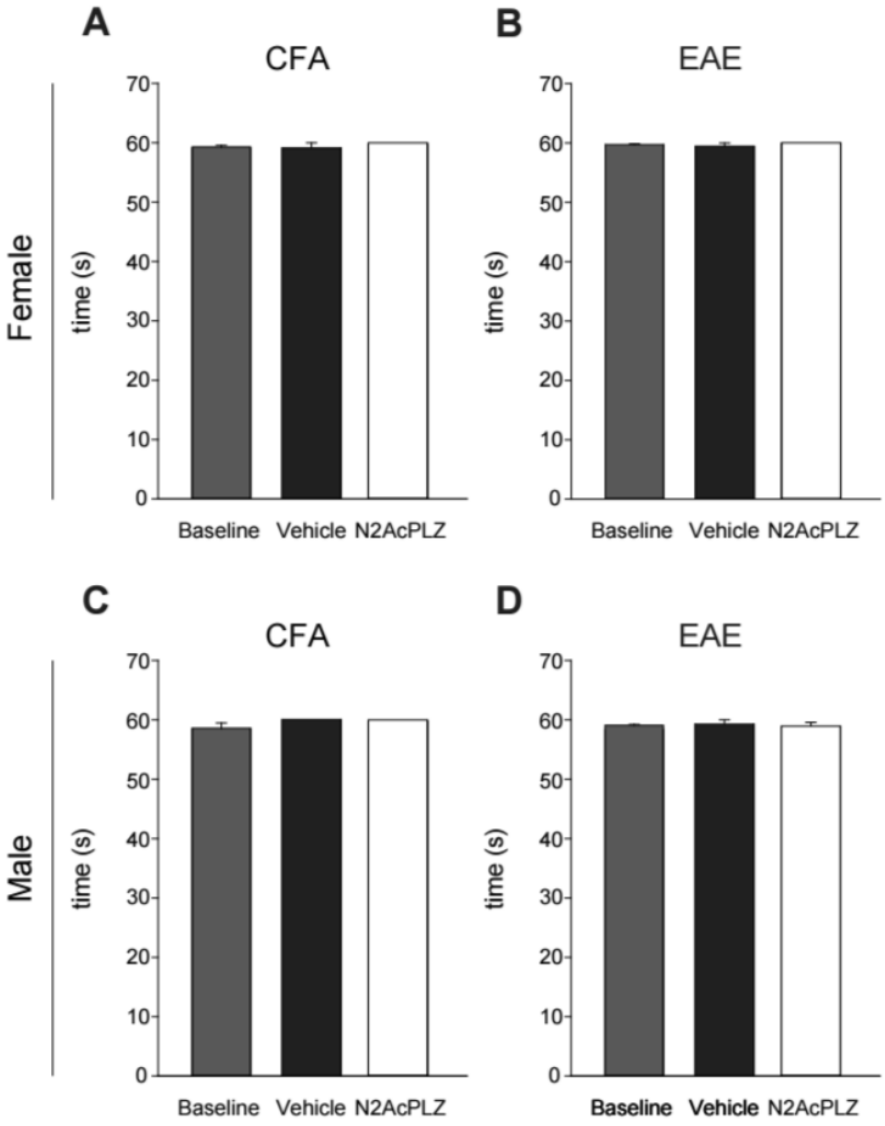

Figure 14. Motor function in mice with EAE was measured using the rota-rod. A, B, C, D) No motor deficits were observed with vehicle or N2-Ac-PLZ treatment in female or male CFA and EAE mice. $*,^{\#}p<0.05$, One-Way ANOVA. Data are means ± SEM.

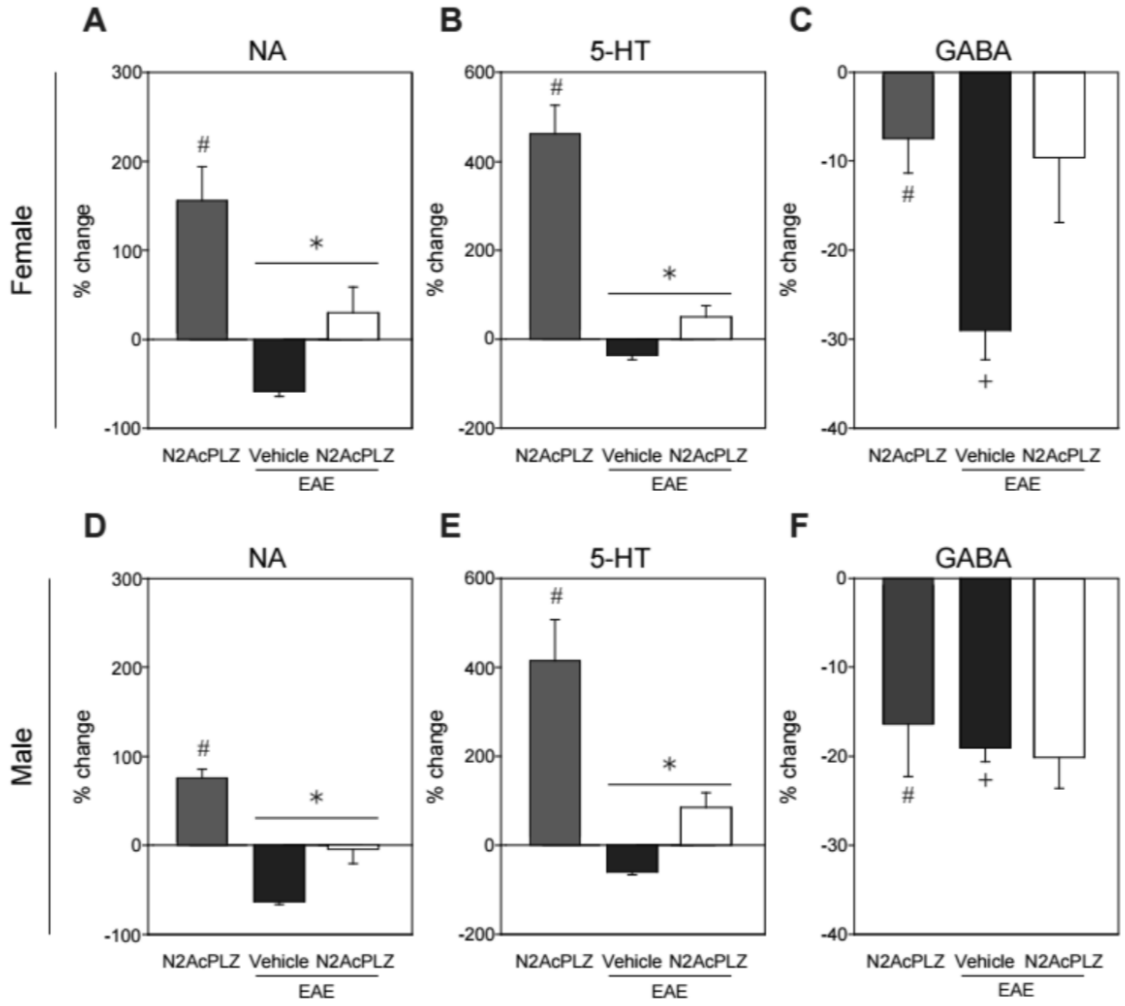

Figure 15. Spinal levels of NA, 5-HT, and GABA in mice with EAE measured with HPLC. Dark gray bars represent CFA mice treated with N2-Ac-PLZ, black bars represent EAE mice treated with vehicle injections, and white bars represent EAE mice treated with N2-Ac-PLZ. All bars represent percent difference from naïve (CFA mice treated with vehicle). A, D) NA levels in female and male mice. N2-Ac-PLZ significantly increased NA levels in both female and male CFA and EAE mice. Overall, females had a larger increase of NA levels with N2-Ac-PLZ treatment than males. B, E) 5-HT levels in female and male mice. N2-Ac-PLZ treatment significantly increased 5-HT levels in female and male CFA and EAE mice. C, F) GABA levels in female and male mice. N2-Ac-PLZ treatment significantly decreased GABA levels in female and male CFA mice when compared to vehicle treated CFA mice. Additionally, GABA levels were decreased in female and male EAE mice treated with the vehicle, also when compared to vehicle treated CFA mice (+). $^{*,\#,+}p<0.05$, Three-way ANOVA followed by *post hoc* Holm-Sidak analysis. Data are means ± SEM.

4. Discussion

4.1 The Present Study

The present study sought to examine the potential therapeutic effects of N2-Ac-PLZ in the EAE model of MS. I assessed the effects of chronic N2-Ac-PLZ treatment on female and male mice with EAE, and evaluated its effects on disease course and exploratory, anxiety-like, and nociceptive behaviours, as well as important neurotransmitter levels within the spinal cord.

4.2 Disease Course

The observation that N2-Ac-PLZ did not significantly alter disease course in mice with EAE of either sex suggests that GABA plays a stronger role in the disease than previously indicated. Previous studies have shown that PLZ administration significantly improved disease severity, and even delayed the onset of EAE in female mice (Benson et al., 2013; Musgrave et al., 2011). To determine whether these beneficial effects on disease outcomes are mediated by the changes in NA/5-HT or GABA I assessed the effects of N2-Ac-PLZ which only elevates the levels of NA and 5-HT. N2-Ac-PLZ treatment did not however, significantly change disease course in either females or males, suggesting that GABA plays a significant role in the development and severity of EAE. I did observe a trend towards a delayed ‘day to onset’ in female mice treated with N2-Ac-PLZ but this effect did not reach statistical significance. Additionally, it should be noted that male mice with EAE had a more severe average peak clinical score than females, suggesting that male’s disease was more severe than females. It is important to realize that the roles of neurotransmitters are extremely complex. Even though previous results seem to indicate that GABA is the primary component differing between PLZ and N2-Ac-PLZ treatment, we should remember that there may be various factors, such as other neurotransmitters and amino acids, that are playing a role in pain development and regulation.

4.3 Exploratory and Anxiety Like Behaviour

The open field test was used to measure exploratory behaviour in mice. In general, total distance moved and time spent in the center zone generally decreased in both naïve and EAE mice after baseline. These results could be due to any number of reasons, but are most likely due to habituation. The mice were placed in the apparatus more than once, therefore might have become familiarized to the once novel environment. Additionally, increased stressors could also be playing a role. The naïve mice received injections prior to testing, which is a stressor, then were housed individually before being tested. This added stress could therefore be contributing to the decreased distance moved and time spent in the center zone. The mice with EAE may have experienced both of these stressors as well, but in addition, experienced the disease which could have affected their desire/motivation to explore a new environment (Ennaceur, Michalikova, van Rensburg & Chazot, 2006).

I used the elevated plus maze (EPM) to measure anxiety-like behaviour in naïve mice, and observed similar results to Musgrave et al. (2011). N2-Ac-PLZ treatment did not significantly increase the number of open arm entries or time spent in the open arms in either sex. However, the data suggest that N2-Ac-PLZ may act to normalize time spent in open arms in both females and males. This may indicate that although the number of entries has decreased, overall the mice are spending more time in the open arms. These results are preliminary, and were only observed in a subset of animals. These findings on the anxiolytic properties of N2-Ac-PLZ in EAE-induced anxiety would need to be replicated in order to make firm conclusions.

4.4 Pain Behaviours: Mechanical Hypersensitivity

The observation that N2-Ac-PLZ treatment improved mechanical hypersensitivity in female mice with EAE offers an opportunity to further explore the underlying mechanisms

behind sex differences in pain. Previous data have shown that PLZ treatment improves mechanical hypersensitivity in female EAE mice (Potter et al., 2016). PLZ, and its derivative N2-Ac-PLZ, have been shown to decrease time spent engaging in nociceptive behaviours in the formalin model in male mice (Benson et al., 2013; Mifflin et al., 2016; Musgrave et al., 2011). Whereas treatment with PEH, which only elevates levels of GABA decreased nociceptive behaviours in both sexes (Mifflin et al., 2016). These findings suggest that GABA plays a strong role in female nociception, whereas males can engage noradrenergic/serotonergic systems along with GABA to modulate nociception.

It is important to note that the formalin model is only a tonic model of pain processing, representing a completely different type of pain. It was important to explore the effects of N2-Ac-PLZ in a model of disease-related chronic pain. In the present study, we found that N2-Ac-PLZ-treated female mice with EAE had significantly higher thresholds in the Von-Frey task when compared to vehicle-treated females at disease onset. The data also showed a trend towards an increase in mechanical thresholds in female CFA control mice who received N2-Ac-PLZ. These results are contrary to what we had initially predicted, based on data from the formalin model, and the question that arises is why males did not benefit from N2-Ac-PLZ.

4.5 Neurotransmitter Levels

As predicted, N2-Ac-PLZ increased NA and 5-HT levels in naïve and diseased mice of both sexes. Unexpectedly, naïve female mice had larger increases of NA and 5-HT levels when compared to naïve males. Furthermore, female mice with EAE had higher levels of NA when compared to males with EAE that were treated with N2-Ac-PLZ. These data did not support the hypothesis that NA and 5-HT levels would be similarly elevated in both the sexes. It should be noted that N2-Ac-PLZ increased levels of NA and 5-HT more in naïve mice than in mice with

EAE. The differences between naïve and EAE mice are likely due to increased stress and pain from injections every other day, as well as coping with a disease that drastically decreases NA, 5-HT, and GABA levels (Musgrave et al., 2011). Therefore, N2-Ac-PLZ treatment may be acting to normalize levels in mice with EAE, whereas it acts to significantly increase baseline levels in naïve mice.

A surprising finding was that N2-Ac-PLZ treatment significantly decreased GABA levels in CFA control female and male mice, although the decreases were small. Lowered GABA levels have been reported as a characteristic of pain, so it is probable that these findings are a consequence of the pain and stress of multiple injections (Jasmin et al., 2003; Zeilhofer, Möhler & Lio, 2009). In general, GABA levels decreased in female and male mice with EAE, with no significant difference between vehicle or N2-Ac-PLZ treated mice. Again, this is likely due to the disease itself, since decreased levels of GABA have been correlated with EAE (Musgrave et al., 2011; Musgrave et al., 2011). It is also possible that N2-Ac-PLZ may be affecting GABA levels indirectly through primarily noradrenergic or serotonergic pathways (Attal et al., 2010; Benson et al., 2015; Mico´, Ardid, Berrocoso & Eschalier, 2006; Dupuis et al., 2016; Eide & Hole, 1993; Mika et al., 2013; Ortega, Fernández-Pastor, Callado & Meana, 2010).

4.6 Mechanism of Action

Although this study provides some interesting information regarding the effects of N2-Ac-PLZ on nociception within females, I was unable to determine the specific mechanism of action by which this happened. Studies have shown that pain develops and is regulated via different neurotransmitter systems in each of the sexes, with GABA playing a significant role in female nociceptive regulation compared to males who seem capable of engaging NA/5-HT and GABA to regulate nociceptive sensitivity. Therefore, if N2-Ac-PLZ were to have antinociceptive

properties we would predict that it would primarily benefit male mice with EAE. Contrary to this hypothesis, my results support the idea that female mice with EAE may not require a GABA component to regulate nociception. N2-Ac-PLZ treatment improved tactile hypersensitivity in female mice with EAE but was ineffective in males. Although my research focused on how NA, 5-HT and GABA levels were affected with N2-Ac-PLZ treatment, it is possible that other neurotransmitters, such as dopamine (DA), were also affected and played a role in the observed results (Jarcho, Mayer, Jiang, Feier & London, 2013). DA has been implicated in the modulation of pain perception, and is supported by evidence demonstrating that drugs which enhance dopaminergic neurotransmission provide analgesia in both rodents and a clinical population (Baliki, Geha, Fields & Apkarian, 2011; Gerdelat-Mas et al., 2007; Jarcho et al., 2013; Pontieri, Tanda & Di Chiara, 1995). Further investigation into N2-Ac-PLZ's effects on other neurotransmitters within the CNS would provide a better understanding of the mechanisms underlying sex differences within pain. These findings suggest that the sex-specific mechanisms that regulate pain behaviours are engaged differently depending on the type of pain (acute, sub chronic and disease-related chronic).

4.6-1 Immune Response

A possible explanation for the results in the EAE model is differing immune responses of female and male mice with EAE. Sex differences are apparent within MS, starting with the majority of patients being female, and experiencing higher levels of neuropathic pain (Orton et al., 2006). Female susceptibility to other autoimmune conditions is also elevated, and this is suggested to be due to women's strong immune response (Dunn et al., 2007; Whitacre, Reingold & O'Looney, 1999). It has been observed that immune cell infiltration differs between the sexes in both patients and animal models (Dunn et al., 2010; Dunn et al., 2007; Mifflin, Frieser,

Benson, Baker & Kerr, 2017; Sorge et al., 2015). It has been suggested that males use microglial-dependent pathways for pain processing, whereas females preferentially use adaptive immune cells but can switch to a glial-dependent mechanism if none are available (Sorge et al., 2015). These differences may be due to the females having larger quantities of T-lymphocytes, such as $CD4^+$ and $CD8^+$ cells, in both the periphery and spinal cord (Scotland, Stables, Madalli, Watson & Gilroy, 2011). Another factor potentially contributing to these observed sex-differences in immune function is a dissimilar expression of peroxisome proliferator activated receptors (PPARs)α and γ in T-cells (Zhang et al., 2012). These different levels of PPARα and PPARγ in T cells in the sexes generate different populations of T-cells between the sexes. Male mice have T cells that are skewed towards a Th17 phenotype (producing higher levels of IL-17) while female T cells are skewed towards a Th1 phenotype (producing higher levels of IFNγ). Whether these specific classes of T cells differentially affect pain sensitivity and/or the responses to treatment is currently being investigated by other members of the Kerr laboratory.

It is worth noting that Benson et al. (2013) found that PLZ treatment did not affect female mice's immune responses in EAE. They measured infiltrating CD4+ T-cells and Iba-1 reactivity, markers for inflammation and microglial/macrophage activation, in mice with EAE treated with PLZ. PLZ had no significant effect on the number of CD4+ T-cells, or the density of Iba-1 in the disease although they reported a delayed disease onset and improved outcomes with PLZ treatment. Since no changes were observed in these immune parameters, it is possible that PLZ could be modulating the phenotype or cytokine profile of the T-cells and/or infiltrating macrophages, therefore improving the disease course and pain hypersensitivity. Other data have shown that increased GABAergic activity improves disease severity in mice with EAE, but also does not directly affect T-cells (Bhat et al., 2010). Based on these data, we can postulate that N2-

Ac-PLZ will not significantly affect the immune response (recruitment or density of cells in the CNS) in either sex in EAE. However, it remains to be determined whether N2-Ac-PLZ can influence the cytokine profile of activated T cells or macrophages, and whether it would impact pain signaling in the disease. If N2-Ac-PLZ can modulate T-cell/macrophage phenotypes, then one would predict that female T-cells and macrophages are more susceptible to this modulation.

4.6-2 Oxidative Stress & Pain

Oxidative stress (OS) may explain the ineffectiveness of N2-Ac-PLZ on hypersensitivity in male mice with EAE. OS is reported to play an important role in many conditions such as MS and chronic pain, although data vary. OS is classified as the imbalance between the production of reactive oxygen species (ROS) and the cell's ability to defend against them (Gilgun-Sherki, Melamed & Offen, 2004). Increased OS carries consequences, such as increasing the CNS's susceptibility to neurodegeneration (Halliwell, 2001). OS is typically controlled by endogenous antioxidant systems, but unregulated ROS generation can disrupt this essential balance (Valério et al., 2009). ROS have been named as a major player in the development of MS, where elevated ROS contributes to invoking an inflammatory response, and increasing pain (Valério et al., 2009). This results in an influx of T cells and macrophages into the CNS which causes considerable damage (Uttara, Singh, Zamboni & Mahajan, 2009). Data has shown that decreasing levels of OS, or inhibiting ROS, in the spinal cord can produce anti-nociceptive effects, and this has become a target in treatment development (Fidanboylu, Griffiths & Flatters, 2011; Valério et al., 2009; Wang et al., 2004). Studies within a clinical population have suggested that oxidative damage, alongside an enhanced immune response, contribute to disease progression in MS patients (Choi, Lee, Hughes, Denney & Lynch, 2016).

There are various means to measure OS, but commonly used ones include using the ratio of gluthathione (GSH) and glutathione disulfide (GSSG) levels and/or the levels of the enzyme superoxide dismutase (SOD) (Choi, Lee, Hughes, Denney & Lynch, 2016). GSH is a critical endogenous antioxidant that acts to protect cells against oxidative damage by detoxifying ROS (Bains & Shaw, 1997; Pompella, Visvikis, Paolicchi, De Tata & Casini, 2003). During detoxification, GSH is converted to its oxidized form (GSSG), therefore as GSSG levels increase, GSH/GSSG ratios decreased, and indicating increased OS (Choi, Lee, Hughes, Denney & Lynch, 2016; Halliwell, 2001). Therefore, when GSSG levels increase, this leads to a reduced GSH/GSSG ratio, and is indicative of increased OS (Lew, Pyke & Quintanilha, 1985; Schafer & Buettner, 2001). SOD, another commonly used marker of OS, plays a role in the conversion of superoxide (O_2^-) radicals into oxygen (O_2) or hydrogen peroxide (H_2O_2). Decreased SOD activity is indicative of increased OS (Zargari et al., 2007).

Within the EAE model, studies have shown that OS and damage differ between females and males. Benson et al. (2015) observed that female mice with EAE who were offered voluntary wheel running (exercise has been reported as a beneficial treatment in MS and EAE) had an elevated GSH/GSSG ratio, indicating lower levels of OS. Previous data have also shown that exercise increased GSH/GSSG ratios in male mice with EAE at a chronic time point (Mifflin et al., 2017). In general, SOD levels and activity have also been found to decrease in EAE (Miller, Walczak, Majsterek & Kędziora, 2013). Mifflin et al. (2017) observed reduced SOD enzyme activity in diseased females, indicating that perhaps females with EAE are unable to use this antioxidant pathway, or resources are used up and exhausted. The results produced by Mifflin et al. (2017) were also accompanied by improvements in clinical disease in both females and males, although unequally. These studies give a little insight into the research that is being

conducted regarding sex differences in OS and show us that even though there are obvious differences between females and males, there are still a lot of questions that remain unanswered. It is imperative that ratios of GSH/GSSG are examined in future research, in order to determine if OS is counteracting any beneficial aspects of N2-Ac-PLZ.

4.7 GABA Decreases

I was unable to determine why GABA levels were generally decreasing, and why N2-Ac-PLZ treatment further decreased these levels in CFA animals, although decreases were small. A possible explanation for decreased GABA levels is that lowered levels have been identified as a trademark of pain, and these mice are generally experiencing pain within this disease model no matter the treatment they are receiving (Jasmin et al., 2003; Zeilhofer, Möhler & Lio, 2009). This may also explain why GABA levels dropped in N2-Ac-PLZ-treated CFA mice. Although the CFA mice did not receive the active EAE induction, they still received injections every other day, and experienced the same stressors as the sick mice, which could contribute to lowered GABA levels (Biggio et al., 1984).

Conclusion

While my study did not provide mechanistic insight on the therapeutic effects of N2-Ac-PLZ, it did offer supporting evidence of sex differences within the EAE model. I have shown that N2-Ac-PLZ effectively decreases mechanical hypersensitivity in female mice at the onset of EAE. Interestingly, I showed that N2-Ac-PLZ acts to increase levels of NA and 5-HT in both female and male spinal cords post-mortem, although not equally as was predicted. The use of the EAE model provides a useful platform in examining the effects of potential therapeutic agents in both disease course and nociceptive behaviours. It is also valuable in determining sex-specific pathways in which hypersensitivity and disease outcome are regulated. Future investigation into

sex differences and the underlying mechanisms that regulate mechanical hypersensitivity within a disease state are important next steps towards tailoring treatments for more efficacious pain relief.

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