Sex Differences in Central Nervous System Plasticity and Pain in Experimental Autoimmune Encephalomyelitis

by

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Abstract

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease with many known structural and functional changes in the central nervous system (CNS). In disease states such as MS, these changes are often abnormal and contribute to symptom development. A well-recognized, but poorly understood, complication of MS is chronic pain. Using the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, our lab recently demonstrated that EAE-related pain behaviours are associated with abnormal changes in cortical plasticity occurring in response to neuropathic pain states. While we have shown that chronic pain in MS is associated with neuroplasticity changes, little is known regarding the influence of sex on the development and maintenance of MS-related pain. This is important to consider, as MS is a predominantly female disease. The current project uses the EAE model to examine sex differences in measures of spinal cord inflammation and plasticity that accompany tactile hypersensitivity.

EAE was induced by injecting 8-10 week old C57BL/6 mice with myelin oligodendrocyte glycoprotein peptide amino acids 35-55 (MOG_{35-55}) emulsified in complete Freund's adjuvant (CFA). At disease onset (indicated by partial tail paralysis), pain behaviours were assessed using von Frey monofilaments and the acetone test. Spinal cord tissue was extracted on day of disease onset and prepared for Golgi-Cox and immunohistochemical staining to visualize features of lamina 4-5 within the dorsal horn, where pain information is processed, integrated and relayed by wide dynamic range (WDR) neurons. The role of biological markers involved in inflammation, neuronal modulation and plasticity within this pain processing region were explored and compared between sexes.

Both male and female EAE mice demonstrated increased frequency and duration of pain behaviours, however this response was often lateralized in females. While EAE onset was accompanied by significant inflammation in both sexes, male EAE mice showing pain responses exhibit an increased presence of axonal damage markers compared to their female counterparts. We found these males also possess a higher proportion of reactive astrocyte staining in the dorsal horn. Interestingly, males (with or without disease) were discovered to have a reduction in integrity of the extracellular matrix relative to females within our region of interest. Presumed-WDR neurons from male mice showing pain responses have significantly longer dendritic branches on average, as elucidated by Golgi-Cox staining. Furthermore, dendritic spine density analysis revealed deep dorsal horn neurons from male EAE mice showing pain are significantly less complex, whereas the opposite was seen from EAE males lacking pain behaviours.

We believe that EAE-related pain hypersensitivity is associated with abnormal neuroplastic changes in the spinal cord dorsal horn, a key pain processing area within the CNS. While we observed substantial inflammatory activity in both sexes, only male EAE mice exhibit robust staining of axonal injury markers and increased dendritic arbourization in morphology of deep dorsal horn neurons. We propose that tactile hypersensitivity in male mice with EAE may rely more heavily on neurodegenerative and plasticity-related mechanisms compared to female EAE mice, in addition to the immune system dysregulation prominent in both sexes. Sex differences in deep dorsal horn neuronal morphology, axonal injury and gliosis in the spinal cord associated with pain early in EAE progression supports the idea of differentially regulated pain pathways between the sexes. Results from this study may indicate future sex-specific targets that are worth investigating their functional role in pain circuitry.

Preface

This research was conducted at the University of Alberta under the supervision and guidance of Dr. Kerr, Dr. Winship, Dr. Colbourne and Dr. Sipione. All work in this project was carried out under approval of the University of Alberta Animal Research Ethics Board. EAE inductions were performed by the Kerr lab. All animal experiments and procedures were conducted in accordance with the Canadian Council on Animal Care's Guidelines and Policies and with protocols approved by the University of Alberta Health Sciences Animal Care and Use Committee (AUP#00000274). My role in this project includes behavioural testing, tissue processing, data collection and analysis, creating and finalizing figure presentation, and manuscript preparation. Some of the research conducted for this thesis was performed in collaboration with members of the Winship lab at the Neurochemical Research Unit in the Department of Psychiatry, University of Alberta. J.W. Paylor of the Winship lab assisted with data analysis in Figure 10 as well as obtaining high resolution confocal images for representative images used in figures. G. Tenorio of the Kerr lab assisted with graph preparation in this thesis. This thesis is original work written by myself, the author, with edits suggested by my supervisory committee. This research received funding support from an MS Society of Canada endMS Studentship Award.

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Thank you to my family and friends who provided a constant stream of love and support – you know who you are. I am especially thankful for my parents and my brother, who listened to me during my stress and doubt. Finally, it seems appropriate to include a quote from a renowned neuroscientist who worked through the trials and tribulations of the Golgi method. I strive to carry this sentiment with me in my future endeavours:

It is fair to say that, in general, no problems have been exhausted; instead, [we] have been exhausted by the problems. Soil that appears impoverished to one researcher reveals its fertility to another. Fresh talent approaching the analysis of a problem without prejudice will always see new possibilities – some aspects not considered by those who believe that a subject is fully understood. Our knowledge is so fragmentary that unexpected findings appear in even the most fully explored topics.

– Santiago Ramón y Cajal, *Advice for a Young Investigator*

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Glossary of Terms & Abbreviations

Amyloid Precursor Protein: APP

Analysis of Variance: ANOVA

Blood-Brain Barrier: BBB

Central Nervous System: CNS

Cluster of Differentiation 4: CD4

Complete Freund's Adjuvant: CFA

4',6-Diamidino-2-Phenylindole: DAPI

Experimental Autoimmune Encephalomyelitis: EAE

Glial Fibrillary Acidic Protein: GFAP

Ionized Calcium-Binding Adapter Molecule: Iba1

Multiple Sclerosis: MS

Myelin Oligodendrocytes Glycoprotein: MOG

Neurofilament H Non-Phosphorylated: SMI-32

Phosphate-Buffered Saline: PBS

Serotonin: 5HT

Standard Error of the Mean: SEM

Vesicular Glutamate Transporter 1: VGlut1

Wisteria Floribunda Agglutinin: WFA

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by inflammation and demyelination. Currently, Canada has one of the highest rates of MS globally (Gilmour et al., 2018). Clinical manifestations of the disease often include cognitive, motor, sensory and autonomic disturbances such as deficits in executive functioning, declined visual acuity and gait ataxia (Compston and Coles, 2008). Symptoms vary in nature and severity, and can be experienced in periods of relapse and remission over the course of the disease. Classification of MS is based on clinical course of the disease, with patients presenting as relapsing remitting, progressive, or progressive relapsing (Hauser and Oksenberg, 2006; Compston and Coles, 2008; Tullman, 2013). Treatments currently used to help manage symptoms of MS include various forms of pharmacological and rehabilitation therapies, which vary in their efficacy with the stage of disease (Gilmour et al., 2018). The heterogeneous nature of MS presentation within the clinical population makes both the treatment and research more difficult.

A well-recognized, but poorly understood, complication of MS is chronic pain. In the general population, chronic pain affects approximately 20% of individuals worldwide (Österberg et al., 2005; Treede et al., 2015). Chronic pain reportedly affects over 50% of MS patients in the course of their disease, which greatly affects quality of life (Archibald et al., 1994; Österberg et al., 2005). MS-related pain is characterized by increased sensitivity to non-painful stimuli (known as allodynia), as well as prolonged pain responses to painful input (hyperalgesia). More specifically, the occurrence of this pain has been described by patients as often bilateral aching, burning and pricking sensations in both the lower and upper extremities (Österberg et al., 2005). To date, there is still no effective treatment for MS-related pain.

1.1 Sex differences in MS

The incidence of MS in Canada is substantially higher in females, outnumbering males as high as three fold (Österberg et al., 2005; Orton et al., 2006). Furthermore, women outnumber men in reports of chronic central pain symptoms across many chronic pain syndromes (Mogil, 2012; Sorge and Strath, 2018). While women are more susceptible to developing relapsing remitting MS, men with MS more often display progressive disease courses, described by rapid progression of disability and poorer recovery from initial attacks (Bove and Chitnis, 2014). Early progression of MS is highly variable, however adverse prognostic signs include older age at onset, male sex, frequent attacks and incomplete recovery from attacks early in disease, and rapid accumulation of progressive disability (Tullman, 2013). Functional connectivity impairments as a result of thalamic atrophy is also notably observed in males, which can contribute to the higher risk of cognitive decline (Schoonheim et al., 2012; Golden and Voskuhl, 2017). Increased susceptibility of females and more rapid progression of disability and cognitive impairment in males suggests differing disease mechanisms. Though the exact mechanisms underlying sex differences in MS are unknown, examples of theories proposed involve hormonal modulation, environmental and epigenetic risk factors. More specifically, a notable increase in MS risk occurs in females following puberty, particularly earlier age of puberty, which supports the role of sex hormones in MS (Chitnis, 2013; Ahn et al., 2015). Furthermore, male onset of MS tends to occur later in adulthood when testosterone levels start to decrease, implying a protective factor of testosterone in men (Pines, 2011; Golden and Voskuhl, 2017). Thus, it is likely there are complex inflammatory or neuromodulatory effects of gonadal hormones in MS and perhaps other demyelinating diseases.

MS disease and susceptibility may also be modulated differentially between sexes by environmental and lifestyle factors such as smoking, vitamin D, uric acid, sunlight and Epstein-Barr virus exposure (Bove and Chitnis, 2013). However, the relationship between these factors and disease progression is complicated and not yet fully understood. Sex-specific gene-environment interactions in MS have also being observed, notably increased risk in females who inherit certain types of the human leukocyte antigen, namely the HLA-DRB1*15 allele, which encode the major histocompatibility complex proteins in humans (Chao et al., 2009; Sadovnick, 2009). Of course, the undoubted complexity of interactions between physiological, cultural and psychological factors prove to be a substantial barrier to studying sex differences in pain mechanisms. Moreover, variations in the onset and duration of pain, use of analgesic medications, and presence of comorbidities provide methodological challenges in studying patients with chronic pain (Racine et al., 2012). It is also important to acknowledge that chronic pain states are often accompanied by functional impairment and emotional distress that can influence subsequent pain management.

Only more recently has emphasis been placed on studying both sexes in basic research settings. In the human MS population, females have greater susceptibility to developing the disease while males often have worse disease progression, which are postulated to be due to sex differences in the immune and nervous systems, respectively (Golden and Voskuhl, 2017). Thus, sex differences in biological mechanisms must be understood in order to facilitate initiatives towards personalized medicine. Recent advances in research using rodent models stress the importance of the immune system that underlie observed biological differences in pain (Sorge and Totsch, 2017). In several preclinical models, female rodents exhibit heightened sensitivity to external stimuli, which have been attributed to a pronounced immune cell response to pathogens

and injury (Golden and Voskuhl, 2017; Sorge and Totsch, 2017; Sorge and Strath, 2018). Interestingly, it has been observed that female and male mice preferentially utilize T lymphocytes and microglia in the spinal cord, respectively, to mediate chronic pain behaviour in a spared nerve injury procedure (Sorge et al., 2015). The interaction of immune cells, glia and neurons in both the periphery and CNS is important to consider, as neither component acts in isolation to produce neuropathic pain states (Scholz and Woolf, 2007). For example, astrocytes and microglia are known to activate following injury and support the transition from acute to chronic pain conditions, a process that is dependent on sensory neurons releasing proinflammatory cytokines (Chiang et al., 2012; Joller et al., 2012; Sorge and Totsch, 2017). This process is perpetuated by further release of inflammatory mediators and chemokines from glia cells, and recruitment of different immune cell populations to mediate response to injury. Sex differences in this triad may greatly impact what we know about initiation of clinical pain states, chronic pain maintenance and treatment outcomes. A greater understanding of how sex affects MS-related pain is needed to provide more effective, targeted treatments.

1.2 EAE and Pain

Chronic pain is a debilitating condition, characterized by pain lasting longer than three months and persists past normal healing time. Chronic pain affects approximately 20% of people worldwide and encompasses a wide range of categories such as headaches, cancer pain and neuropathic pain (Treede et al., 2015). Neuropathic pain may arise as a result of damage or lesion of the somatosensory nervous system, and often includes critical involvement of the immune system (Scholz and Woolf, 2007; Treede et al., 2015). A commonly used mouse model of MS, experimental autoimmune encephalomyelitis (EAE), can be utilized to study the association between pain behavior and structural changes in the nervous system (Kipp et al.,

2012; Rangachari and Kuchroo, 2013). EAE is a T cell-mediated autoimmune disease that attacks endogenously expressed myelin proteins in the CNS. EAE mimics the chronic progression of MS and results in hallmark pathologies characteristic of MS such as axonal demyelination and inflammation in the CNS (Goodin, 2014; Robinson et al., 2014).

Our lab, in addition to the work of many others, have repeatedly demonstrated that the EAE model displays increased pain behaviours (e.g. tactile hypersensitivity) and pathophysiology similar to those observed in people with MS (Olechowski et al., 2009; Potter et al., 2016; Thorburn et al., 2016). Induction of EAE involves immunization with synthetic myelin, neural or glial antigens, often emulsified in an adjuvant such as complete Freund's adjuvant (CFA) (Kipp et al., 2012). Subsequent intraperitoneal injections of pertussis toxin facilitate the immune response through breakdown of the blood-brain barrier (BBB) (Linthicum et al., 1982). Clinical presentation of this model can vary depending on the animal species and strain, immunization methods used as well as the peptide used. In the present study, myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) is used, as this peptide is known to induce a chronic and progressive disease course in C57BL/6 mice (Rangachari and Kuchroo, 2013; Robinson et al., 2014). Signs used to assess the disease in mice involve gradual progression of hind limb paralysis, beginning at the tail. Changes in motor functioning is apparent at the level of the spinal cord by immune cell infiltration and widespread axonal damage and loss (Robinson et al., 2014).

Pain behavior can be measured in EAE using a variety of standardized assays that assess modalities such as tactile and temperature sensitivity. An increasing body of literature has demonstrated that EAE mice exhibit increased nociceptive behaviours such as grimacing, tail flicking and paw shaking when clinical signs emerge (Aicher et al., 2004; Khan and Smith, 2014; Grace et al., 2017). At disease onset, the first day mice exhibit clinical signs of EAE, thresholds

to mechanical stimuli using von Frey monofilaments are reduced in comparison to baseline scores prior to EAE induction (Olechowski et al., 2009). Furthermore, EAE mice also demonstrate prolonged responses in the acetone test and increased anxiety-like behaviours (Olechowski et al., 2009, 2013; Potter et al., 2016). While there is no perfect animal model to study chronic pain in MS, the EAE model allows for increased insight into MS pathology and the mechanisms that serve to protect from or drive chronic pain states. The varying EAE models accommodate the heterogeneous clinical population of MS and can be used to study disease progression, histopathologies of MS-like conditions and development of potential therapeutic interventions. Using the EAE model to gain a greater understanding of spinal circuitry in chronic pain states provides an opportunity to identify novel, sex-specific intervention strategies.

1.3 Plasticity in Pain

Neuroplasticity is a highly dynamic process that involves constant remodeling, strengthening or weakening of neural connections in response to neural activity and other neuronal stimuli. Plasticity can be inferred by changes in gene and protein expression, electrophysiology, cortical organization and synaptic connections, which may ultimately reflect changes in behavior (Kolb and Teskey, 2012). Synaptic connections between neurons provide the basic mechanism for communication in the nervous system. Studying neuronal morphology can provide clues about the functional organization of a particular area of the CNS as well as the cells that reside in that area. Observing specific regions of any given neuron may further our understanding of the neuron's targets and inputs via the axonal projections and dendritic arbour, respectively. Furthermore, dendritic spines are postulated to be involved in many plasticity-related processes, as they regulate and translate synaptic activity into morphological changes (Segal, 2010). Dendritic arbourization and spine organization has been extensively studied and

identified as integral components in modulating experience-dependent change in the nervous system, such as learning and memory (Kolb and Whishaw, 1998; Alvarez and Sabatini, 2007). However, dendritic plasticity is also implicated in states of injury, stress and chronic pain. Previous work has demonstrated maladaptive functional implications of altered dendritic spine density and morphology on dorsal horn neurons in nerve injury and diabetic neuropathy models (Tan et al., 2009, 2012). Changes in dendritic spine and branching morphology may deviate from healthy functioning in neuronal circuits mediating pain processing via loss of inhibitory control and aberrant neuro-immune interactions (Labrakakis et al., 2011).

In disease states, the capacity to alter connectivity and communication between cells is often abnormal and can contribute to symptom development. Furthermore, when abnormal rearrangements between connected cells in the brain and spinal cord occur, chronic pain states may arise (West et al., 2015; Cordero-Erausquin et al., 2016). Although previous work in our laboratory has linked pain behaviour to changes in inflammation, we have recently demonstrated that EAE-related pain behaviours are also associated with functional and structural changes (i.e. neuroplasticity) in the brain. Specifically, we found that somatosensory cortex activity evoked by sensory stimulation is enhanced in EAE mice *in vivo*, demonstrating functional plasticity (Potter et al., 2016). We also found evidence of increased neuronal sprouting in the primary somatosensory cortex, demonstrating structural plasticity. These findings corroborate research showing that abnormal changes in cortical plasticity occur in response to neuropathic pain states (Gustin et al., 2012; Nistico et al., 2014). This altered plasticity may then contribute to the cognitive impairment, enhanced neurodegeneration and prolonged pain states seen in MS. Neuroplasticity changes are likely significant contributes to EAE- and MS-related pain and may be viable targets for novel analgesic drugs and therapies.

The dorsal horn of the spinal cord receives a wide array of sensory information from the environment and modulatory inputs from higher centers. Integration of this information such as touch, vibration, pressure and pain are sent to supraspinal areas that ultimately lead to perception of sensation. Neuronal networks within the spinal cord are complex and dynamic. The gray matter of the spinal cord is organized into laminae, or areas of distinct cellular populations and characteristics, which was first described in cats (Rexed, 1952). Laminae 1-6 in mice make up the dorsal horn where pain information is received from the periphery (Sengul and Watson, 2012). Traditionally, painful stimuli is thought to relay into superficial laminae 1-3 in the dorsal horn via unmyelinated C-fibers and a subset of thinly myelinated Aδ-fibers (Braz et al., 2014). However, nerve injury models have demonstrated nociceptive information may also be carried by Aδ- and Aβ-fibers and processed in deeper laminae, 4-6, where interneurons and wide dynamic range (WDR) projection neurons reside (Heise and Kayalioglu, 2009; Gangadharan and Kuner, 2015). WDR neurons contain complex receptive fields and receive both noxious and innocuous stimuli across all somatosensory modalities (Woolf and King, 1987; De Koninck et al., 1992). Characteristic of these convergent cells is their increased action potential firing frequency as intensity of peripheral stimulation from Aα, Aβ, Aδ and C-fiber afferents increases, which is often associated with neuropathic pain states when action potential generation becomes abnormal (Mendell, 1966; Waxman and Hains, 2006). Furthermore, WDR neurons relay this wide array of information through the spinothalamic tract, the major ascending nociceptive pathway, projecting to the thalamus (Mendell, 1966; Martin et al., 1990).

The processing of nociceptive stimuli is proposed to be lateralized, as clinical evidence supports reports of heightened sensitivity to painful stimulation on the left side of the body in humans (Merskey and Watson, 1979). Reduced pain thresholds on the left side of the body is

subsequently accompanied by asymmetrical brain activation of the right hemisphere, confirmed in a variety of imaging studies (Coghill et al., 2001; Klemenz et al., 2009). Furthermore, the lateralized modulation of pain is supported by evidence of heightened activity in the right prefrontal cortex during the anticipation of painful stimuli (Benedetti et al., 2005). One interpretation is that the right hemisphere is both dominant in emotional processing, yet less efficient in processing somatosensory input than the left hemisphere (Merskey and Watson, 1979; Coghill et al., 2001). Lateralized pain perception, amongst other variables identified in this study, further contributes to the complexity of the origins of chronic pain. Studying sex-specific differences in pain-processing neurons and interlaminar connections within the dorsal horn may depict chronic pain states in a different light from what is known today. A greater understanding of spinal circuits provides opportunity of novel intervention strategies to relieve pain states and other conditions.

1.4 Present Study

To date, our lab has primarily used female, but not male, mice to understand the relationship between EAE-related CNS plasticity and pain. This is an important distinction because several lines of evidence indicate that the mechanisms underlying chronic pain are different between males and females (Mogil, 2012). A more thorough understanding of sex differences in MS-related pain mechanisms is important for developing targeted, sex-specific treatments. The present study investigates the relationship between sex, inflammation, and plasticity within the pain processing regions in the spinal cord of EAE mice. The EAE model allowed us to observe changes occurring at a cellular level at onset of disease, when pain behaviours often emerge. Furthermore, the potential effect of reproductive hormones on pain in females at different phases of the estrus cycle was taken into consideration.

One cohort of animals was dedicated to studying sex differences in pain and immunohistochemical markers of inflammation, demyelination and axon injury. Immunohistochemical staining allowed us to visualize environmental features within the dorsal horn where WDR neurons reside. The aim of this portion of the study was to observe different components of an inflammatory environment that could potentially interact with and modulate morphological changes in pain-processing deep dorsal horn neurons, specifically at onset of disease.

The second experiment specifically focused on morphological features of presumed-WDR neurons at onset of disease in the dorsal horn. I assessed measures of plasticity such as dendritic branching and spine density on deep dorsal horn neurons in mice displaying pain behaviours. In this study, plasticity is defined as a difference, either an increase or decrease, in dendritic arbourization or spine density when compared to morphologically similar cells and areas of similar cell density in CFA control animals. Golgi-Cox staining methods used allow for reliable visualization of neurite processes, namely the dendritic tree. Well-described evidence suggests that neuroplasticity changes occur in response to injury and stress (Kolb and Whishaw, 1998). However, current studies have not yet characterized neuroplasticity changes in the spinal cord in EAE-related pain between sexes, though we know there are prominent neuroinflammatory changes in these areas (Bradl and Hohlfeld, 2003). An improved understanding of the relationship between sex, neuroplasticity and pain in MS may lead to the development of novel analgesics and therapies for MS-related chronic pain.

2. Methods

2.1 Subjects and Experimental Testing. All animal experiments and procedures were conducted in accordance with the Canadian Council on Animal Care's Guidelines and Policies and with protocols approved by the University of Alberta Health Sciences Animal Care and Use Committee (AUP#00000274). This study aims to describe animal research methods and results according to reporting standards outlined by (Kilkenny et al., 2010). In total, female (N=40) and male (N=35) C57BL/6 mice aged 6-8 weeks old were received from Charles River Canada Laboratory in QC, Canada. One cohort of mice was used for Golgi-Cox staining methods and a second for immunohistochemistry (described as Cohort 1 and 2 in Table 1, respectively). Mice excluded from this study are listed in Table 2. Mice were housed in wire-top cages (5 mice per cage) with free access to water and standard food pellets. Mice were kept in an animal facility monitored room with controlled 12-hour light and dark cycles. All experimental procedures were performed during light cycles only. Prior to behavioural testing or any handling, mice were allowed an acclimation period of 2-3 days upon arrival to the animal housing facility. A subsequent 2-3 days was spent handling the animals in the testing room to ensure familiarity of the experimenter. Mice were distinguished from their cage mates with tail markings using non-toxic permanent markers. Following baseline testing periods, cages were randomly assigned to control (Complete Freund's Adjuvant, CFA) or experimental (EAE) groups.

2.1-1 Immunohistochemistry Experiment

2.1-1.1 Subjects, Treatment and EAE Induction. Female (N=20) and male (N=15) C57BL/6 mice were used for this experiment. To induce EAE, twenty-five mice (15 females, 10 males) received a 50μg subcutaneous injection in the hind flank of myelin

oligodendrocyte glycoprotein peptide amino acids 35-55 (MOG_{35-55}; Stanford Protein and Nucleic Acid Facility, USA) emulsified in CFA (Sigma-Aldrich, Canada) at a final concentration of 1.5 mg/ml. The remaining ten mice (5 females, 5 males) received subcutaneous injections in the hind flank of CFA only (1.5mg/ml) and were considered control. On the day of induction as well as 48 hours later, all mice received intraperitoneal injections of 300 ng of pertussis toxin (List Biological Laboratories, USA) in order to facilitate an immune response. Throughout the experiment, mice were monitored daily for onset of clinical signs of EAE, indicated by a flaccid tail. EAE was scored 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 impaired righting reflex; Grade 4, paralysis of one or both hindlimbs (Kalyvas and David, 2004). All EAE analyses in this study refer to the onset time point, or Grade 1, only. No animals progressed past clinical Grade 1 and therefore do not exhibit any hindlimb weakness, paralysis, or righting reflex impairment.

2.1-1.2 Behavioural Testing. Baseline testing began at least one week prior to assignment of mice to treatment groups. During testing periods, male and female cages were separated to minimize additional stress. All mice were allowed 10 minutes in the testing apparatuses to gain familiarity with testing environments. All testing times were alternated between a set morning or afternoon session to ensure males and females were tested equally in terms of time of day. Following baseline testing and EAE inductions, mice will have reached the age of sexual maturity when sex differences may emerge.

2.1-1.2.1 Mechanical Hypersensitivity. Von Frey monofilaments were used as a behavioural assay to measure tactile hypersensitivity of the hind paw as described

previously (Olechowski et al., 2009). Each filament consists of an elastic column that elicits a specific force in grams (ranging from 0.07 g to 2.0 g) when manually applied to the plantar surface of the hind paw. Withdrawal of the hind paw by shaking, lifting or licking was recorded as a nociceptive response to the applied stimulus. Mice were placed individually in plexiglass chambers suspended on wire mesh for the experimenter to gain access underneath the hind paw. The pain threshold during each testing session was determined by the lowest force applied to elicit at least three nociceptive responses out of five total repetitive stimuli. A smaller amount of force required to elicit nociceptive responses are considered to indicate hypersensitivity. Three baseline testing sessions were performed and averaged prior to EAE induction and experimental testing was performed on day of disease onset. During all testing sessions, both paws were tested and received separate scores. Similar to humans with MS, not all EAE mice present with nociceptive behaviour. In order to address the development of a pain phenotype in this assay, we created criteria to categorize the presence or absence of significant nociceptive responses (labeled responders or non-responders, respectively). This criteria is based on previous observation using this assay, where EAE mice commonly exhibit a significant reduction in response threshold (Olechowski et al., 2009; Benson et al., 2015; Potter et al., 2016). Mice were classified as ‘responders’ if their score from one or both paws measured on day of onset/euthanasia decreased by two or more filaments from average baseline score (for example, baseline and onset scores of 2.0g and 1.0g, respectively). Mice whose von Frey scores did not meet this criteria were considered ‘non-responders’, meaning they did not demonstrate a meaningful decrease in withdrawal responses. Data in Figure 1 includes the mean of all animals (responding and non-responding) to depict the global nociceptive trend

in EAE. All subsequent immunohistochemical stain analyses of EAE groups include von Frey responder animals only, as non-responder group sizes of both sexes in Cohort 2 were too small to perform statistical analyses. Information regarding number of responders and non-responders in each cohort is outlined in Table 1.

2.1-1.2.2 Motor Function. The Rotarod apparatus consists of a horizontally-oriented rotating rough cylinder suspended over a platform (Harvard Apparatus, USA). The objective of this assay is to measure gross locomotor ability, by measuring the amount of time spent on the bar without falling (Olechowski et al., 2009). Mice were individually placed on the bar, fixed at 12 rpm, and tested for three consecutive trials of 60 seconds each. Trials were then averaged within each testing session. Not only does a longer amount of time spent on the bar indicate intact motor functioning, it also suggests that a mouse with a high von Frey threshold is not exhibiting a lack of responding due to hind limb paralysis. All mice underwent three baseline as well as day of disease onset testing sessions, after testing with von Frey monofilaments.

2.1-1.3 Tissue Collection and Preparation. On the day of clinical signs of disease presentation (Grade 1), mice were tested using von Frey monofilaments and rotarod. Following testing, mice were euthanized using an intraperitoneal injection of Euthansol (sodium pentobarbital, 340 mg/mL). During sedation, vaginal cytology samples were collected. All mice were transcardially perfused with 0.9% saline solution followed by a 4% paraformaldehyde in 0.1 M phosphate buffer solution. Lumbar spinal cord tissue was extracted and post-fixed at 4°C overnight followed by two days of 30% sucrose solution in 0.1 M phosphate buffer. On day 3 after extraction, tissue was transferred into Tissue-Tek Optimal Cutting Temperature compound, frozen in dry ice and stored at -80°C.

2.1-1.3.1 Estrus Cycle Monitoring. Vaginal lavage was performed on day of euthanasia while mice were sedated. Cytological samples were collected only once in order to reduce invasiveness and stress, which can confound cytological assessments (McLean et al., 2012). The end of a 100 μL sterile pipette tip filled with bacteriostatic water was placed and aspirated at the opening of the vaginal canal until sufficient sample was obtained. Samples were dispensed onto a glass slide and allowed to dry overnight inside of a fume hood. The next day, estrus smears were stained with Crystal Violet for 1 minute, washed with ddH_20 for 2 minutes and coverslipped with Organo/Limonene mounting medium. Ratio of cell types were observed under light microscopy to determine estrus stage of each mouse at the time of sample collection (McLean et al., 2012). Proestrus is dominated by primarily round, nucleated epithelial cells. Estrus contains mostly sheath-like cornified squamous epithelial cells. Metestrus contains mostly leukocytes and some cornified squamous epithelial cells. Diestrus contains a mixture of leukocytes, cornified squamous and round nucleated epithelial cells.

2.1-1.4 Immunohistochemistry. Tissue was sectioned with a cryostat at 20 μM thickness and mounted onto glass slides. Tissue sections were washed 3 times for 10 minutes each in 1X phosphate-buffered saline (PBS) then blocked for 1 hour in PBS with 0.2% triton and 10% goat or donkey serum. Slides were then incubated at room temperature overnight in primary antibody solutions containing 1X PBS, 0.02% bovine serum albumin, 0.02% goat or donkey serum and appropriate antibody concentrations. The next day, tissue was washed as previously described before appropriate secondary antibodies conjugated to Alexa 488, 594 or Streptavidin 647 (1:200, Life Technologies, Canada) were applied for 45 minutes in the dark at room temperature. Myelin was stained by 3 washes for 10 minutes each in 1X

PBS and incubated with Alexa 488-conjugated Fluoromyelin (1:500, Life Technologies) in 0.2% triton and 10% goat serum for 45 minutes at room temperature in the dark. All slides were washed and coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Canada). Primary antibodies and their concentrations used were the following: rat anti-cluster of differentiation 4 (CD4) (1:200, Bio-Rad, USA), rabbit anti-ionized calcium-binding adapter molecule (Iba1) (1:500, Wako, USA), rabbit anti-amyloid precursor protein (APP) (1:1000, Abcam, USA), rat anti-glial fibrillary acidic protein (GFAP) (1:1000, Invitrogen, Canada), guinea pig anti-vesicular glutamate transporter 1 (VGlut1) (1:2000, Millipore, Canada), rabbit anti-serotonin (5HT) (1:5000, Sigma-Aldrich, Canada), mouse anti-neurofilament H nonphosphorylated (SMI-32) (1:500, BioLegend, USA), and biotinylated Wisteria Floribunda Agglutinin (WFA) (1:1000, Vector Laboratories, Canada).

2.1-1.5 Image Acquisition and Analysis. All image acquisition and analysis was performed blind to sex and experimental condition. At least 3-5 images were obtained of both hemispheres of the spinal cord and numeric data was averaged within each animal during analysis. For each given immunohistochemical stain, only sections with identifiable nuclei were analyzed. Images for analysis were acquired using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) with either 5X, 10X, 20X or 40X objective lenses. Analysis of images was performed using NIH ImageJ/FIJI (a distribution of ImageJ) (Schindelin et al., 2012; Jensen, 2013). Jensen (2013) describes methods to quantify histological staining, such as thresholding staining intensity and region of interest selection, which were used in this study (Jensen, 2013). Representative confocal images were taken using a Leica DMI400B microscope (Leica Microsystems, Germany) with a

40X objective lens. Myelin lesions were outlined according to loss of Fluoromyelin staining and accumulation of DAPI nuclei, and calculated as a percent of total white matter area in the spinal cord cross sections to represent percent demyelination (Thorburn et al., 2016). The number of lesions identified per section analyzed were averaged within each animal. APP was qualitatively assessed by manually counting distinct clusters of staining in the white matter. Both myelin and APP was assessed using 5-times magnification images to visualize the entire spinal cord cross section. CD4- and SMI-32-positive cells with DAPI-labeled nuclei were manually counted from 20-times magnification images and expressed as cell density per area analyzed. In order to objectively differentiate SMI-32-positive cells from diffuse staining, a staining intensity threshold that selectively identified concentrated staining surrounding the cell nuclei was applied to images. Only cells highlighted by thresholding were included for cell counts, and the same threshold was applied to all groups. Iba1, GFAP, 5HT and WFA stains were assessed from 10-times magnification images by measuring either the area fraction (Iba1, GFAP and 5HT) or mean brightness (WFA) of stain occupied within the region of interest. For VGlut1 analysis, 40-times magnification images were used to quantify synaptic puncta. Binarized z-projected image stacks were further processed using a watershed tool to measure VGlut1-positive puncta within the region of interest. All tissue sections analyzed were within the lumbar enlargement in reference to the Allen Spinal Cord Atlas (Allen Institute, 2008). For each stain, imaging and analysis parameters were kept constant. Furthermore, with the exception of whole spinal cord section analysis (myelin and APP), all stains were analyzed either in the superficial (lamina 1-3) or deep (lamina 4-5) dorsal horn, or both. For all immunohistochemical stains, only von Frey responder animals (lateral and bilateral) were

analyzed (N=15 female mice, which includes 5 CFA and 10 EAE; N=13 male mice, which includes 5 CFA and 8 EAE; see Table 1).

2.1-1.6 Statistical Analysis. All statistical analyses were performed using Sigmaplot software version 12.0 (Systat Software, Inc., San Jose, CA). Tests used include two-tailed student's t-tests, one-way analysis of variance (ANOVA), two-way ANOVAs and two-way multiple comparison ANOVAs. Post-hoc analyses such as Tukey's test were performed if necessary. Where normality or heterogeneity assumptions are not met, non-parametric tests (ANOVA on ranks) were used. Difference scores in Figure 1A-B were calculated by subtracting each animal's baseline score from their respective disease onset score (or day of euthanasia for CFA). Data in figures are presented as mean ± standard error of the mean or as a percentage where applicable. Significance was set at $P < 0.05$.

2.1-2 Golgi-Cox Staining Experiment

2.1-2.1 Subjects, Treatment and EAE Induction. Female (N=20) and male (N=20) C57BL/6 mice were used for this experiment. All mice received the same acclimation periods as well as EAE induction methods as outlined in *2.1-1.1 Subjects, Treatment and EAE Induction.* In total, twenty mice (10 females, 10 males) were randomly assigned to the EAE group and the remaining twenty mice (10 females, 10 males) assigned to the CFA group. All mice during this experiment were monitored until the day of onset of disease.

2.1-2.2 Behavioural Testing. All testing followed the same protocols as described in **2.1-1 Immunohistochemistry Experiment**, with the addition of the acetone test.

2.1-2.2.1 Mechanical Hypersensitivity. All mice underwent three baseline testing sessions as well as on day of disease onset. Testing between male and females were alternated in the morning versus afternoon as earlier described. Responder criteria was determined as

previously described in *2.1-1.2.1 Mechanical Hypersensitivity.* Data in Figure 1 includes the mean of all animals from both cohorts to depict the global nociceptive trend in EAE. All subsequent Golgi-Cox stain analyses performed referring to responder and non-responder animals are based on the von Frey behavioural assay only. Non-responder group sizes of females were too small to perform statistical analyses, however male EAE mice in Cohort 1 allowed for exploratory subgroup analysis of responders versus non-responders (N=5 animals in each group, see Table 1).

2.1-2.2.2 Temperature Hypersensitivity. The acetone test was used as a behavioural assay used to measure nociceptive behaviours to cold temperatures (Olechowski et al., 2009). The amount of time spent attending to the hind limb (shaking, flicking, licking) within a one-minute timeframe following a drop of acetone applied to the hind paw was considered as a nociceptive response. An increased duration of attending behaviours was considered a hypersensitivity to the stimulus. Mice were placed individually in plexiglass chambers with wire mesh sides to allow access to the hind limb. One drop of acetone was applied to the surface of each paw, one at a time, using a blunted needle to allow for precise application. Mice were given at least one minute between trials for a total of three trials per paw per testing session. Similar to von Frey monofilaments, three baseline tests were performed as well as experimental testing the day clinical signs appeared, prior to euthanasia. Mice were categorized as responders or non-responders by their duration of nociceptive responses recorded, based on average EAE response times previously exhibited in this assay (Olechowski et al., 2009). Mice were classified as ‘responders’ if their time recorded on day of onset/euthanasia increased by an average of 2 seconds compared to average baseline scores. The acetone test was not used to determine laterality in nociception, nor was it used

as classification for responder versus non-responder animals during Golgi-Cox staining analyses, but rather to demonstrate the various sensory modalities affected in EAE. Data in Figure 1 includes means of all animals (responding and non-responding).

2.1-2.2.3 Motor Function. Mice underwent three baseline tests as well as on day of disease onset, similarly described in *2.1-1.2.2 Motor Function.*

2.1-2.3 Tissue Collection and Preparation. On the day disease onset signs appear, mice were tested using von Frey monofilaments, the acetone test and rotarod. Following testing, mice were euthanized using an intraperitoneal injection of sodium pentobarbital. During sedation, vaginal cytology samples were collected. All mice were transcardially perfused with 0.9% saline solution. Lumbar spinal cord tissue was extracted, rinsed with double distilled water and immersed into pre-prepared impregnation solution overnight ("Solution A/B", FD Rapid GolgiStain Kit from FD Neurotechnologies, MD, USA). Impregnation solution was replaced the following day and tissue was stored in the impregnation solution in the dark at room temperature for 2 weeks. Tissue was transferred into a tissue-protectant solution ("Solution C", FD Rapid GolgiStain Kit), for a subsequent 72 hours with the solution replaced once after the first 24 hours.

2.1-2.3.1 Estrus Cycle Monitoring. Vaginal lavage and cytological assessment was performed as previously described in *2.1-1.3.1 Estrus Cycle Monitoring.*

2.1-2.4 Golgi-Cox Staining. Following tissue impregnation, spinal cords were embedded in agarose and cut into 200 μm sections using a vibratome (Leica VT1200S). Free-floating sections were transferred onto gelatin-coated slides and allowed to dry in a slide box at room temperature in the dark overnight. Slides were washed twice for 4 minutes each in double distilled water, incubated in the working stain solution ("Solution D/E", FD Rapid

GolgiStain Kit) for 10 minutes, then washed twice more. Slides are then dehydrated in an ethanol gradient followed by xylene clearing and coverslipped with Permount mounting medium. Whenever possible, slides were protected from light.

2.1-2.5 Image Acquisition and Analysis. Tissue was first observed under bright-field illumination using a Leica TCS SP5 MP confocal microscope and 20X water-immersion objective lens. Neurons in lamina 4/5 of the dorsal horn were located by reference to an annotated atlas (Allen Institute, 2008). These neurons were identified and selected for analysis based on the following criteria (Woolf and King, 1987; Tan et al., 2008a; Tan and Waxman, 2012): 1) The cell soma diameter is 20-50 μm, 2) The cell soma is contained within Rexed lamina 4/5, 3) At least one dendritic projection must cross into an adjacent lamina with respect to the cell soma, 4) More than half of the dendritic projections are contained within the tissue section and appeared to taper at their ends, and 5) Golgi-staining appears to be continuous. All imaging for analysis was performed blind and using confocal reflectance mode (488 nm argon laser, 30/70 R/T filter) from the same microscope. Z-stack images were taken in order to encompass the entire dendritic arbour (2048x2048 pixels, 240x240 nm pixel size, 2X line/frame averaging, 0.54 μm z-length). At least 4-5 images per hemisphere of each animal were acquired and averaged within each animal (N=17 female mice, which includes 10 CFA and 7 EAE tactile responders; N=20 male mice, which includes 10 CFA and 10 EAE, 5 of which are tactile responders; see Table 1). Apart from male subgroup analyses, only von Frey responder animals (lateral and bilateral) were analyzed.

2.1-2.5.1 Dendritic Branching and Sholl Analysis. For each image, neuronal dendritic arbours were reconstructed using the Simple Neurite Tracer plugin in FIJI (Longair et al.,

2011). Traces of each cell provided information regarding dendritic segment lengths and number of branches. Sholl analysis plugin was also applied to the reconstructions to find the number of branch intersections on concentric 50 μm circles relative to the cell soma. Data from all cells were averaged within their respective animal.

2.1-2.5.2 Dendritic Spine Density Analysis. For each animal, 5 neurons per hemisphere were traced according to the same selection criteria mentioned in *2.1-2.5 Image Acquisition and Analysis.* Branches that were traced also required at least a tertiary branch and at most a septenary branch to ensure morphological consistency. Tracing was done manually via Camera Lucida using a 40X objective lens. Dendritic spines were manually counted on each branch traced using a 100X oil-immersion objective. Cells included for analysis were not obstructed such that branch paths were indistinguishable from other cells. Only dendritic spines with clearly identifiable necks were counted for spine density measures. Spine density of each dendritic segment was calculated as the total number of spines divided by the length of the branch. One branch per cell was analyzed, and data from all cells were averaged within each animal. Apart from male subgroup analyses, which compares tactile responders to non-responders, only von Frey responder animals (lateral and bilateral) were included for analysis. The sample size of the female tactile non-responder group was not large enough to perform statistical analysis of a female subgroup comparing tactile responders and non-responders. Responding criteria was defined by tactile pain criteria only.

2.1-2.6 Statistical Analysis. All statistical analyses were performed as described in *2.1-1.5 Image Acquisition, Analysis and Statistics.*

3. Results

3.1 Morality and Exclusions. Group sizes and exclusions are summarized in Tables 1 and 2, respectively. In the Golgi-Cox experiment, two female EAE mice did not reach onset of EAE. In the immunohistochemistry experiment, one female and one male died during EAE induction and two female EAE mice did not reach onset of EAE. In total, three bilateral non-responding female EAE mice were excluded from analyses as the sample size of these groups were not large enough to statistically analyze in their respective cohorts (one in Cohort 1 and two in Cohort 2).

3.2 Disease Course and Day to Onset. Following EAE inductions, mice were monitored daily to assess for signs of disease according to the following clinical score: Grade 0, healthy mouse; Grade 1, flaccid tail; Grade 2, hind limb weakness with normal righting reflex; Grade 3, severe hind limb weakness with slow righting reflex; and Grade 4, paralysis in one or both hind limbs (Kalyvas and David, 2004). The day at which mice reached onset of disease, Grade 1, was noted and compared between females and males. Mice that did not reach Grade 1 were excluded from this study (Table 2). The number of days taken for female and male EAE mice to reach onset of EAE did not significantly differ ($P = 0.073$).

3.3 Most EAE Mice Exhibit Nocicepive Behaviours at Disease Onset. To explore the relationship between sex and pain in EAE, two behavioural modalities were used to examine nociceptive behaviour at onset of disease. It is worth noting that the time of day mice were tested (morning versus afternoon) did not affect scores in either von Frey ($P = 0.69$ in males, $P = 0.72$ in females) or acetone test ($P = 0.88$ in males, $P = 0.77$ in females) assays. The distinction between unilateral and bilateral responder animals was classified based on mechanical hypersensitivity only.

The von Frey monofilaments assay was used to determine mechanical hypersensitivity in both sexes of EAE and CFA mice. Withdrawal threshold comparisons in Figure 1A are expressed as a difference from each respective animal's baseline score, which did not significantly differ between CFA and EAE mice in either females (P = 0.0862) or males (P = 0.225). EAE induction significantly reduced withdrawal thresholds in both female (two-tailed t-test, $t_{33} = 7.374$, P = <0.001; Fig 1Ai) and male mice (two-tailed t-test, $t_{32} = 8.223$, P = <0.001; Fig 1Aii) overall compared to CFA animals. Reduced withdrawal thresholds in both sexes of EAE mice suggest these mice are experiencing tactile hyperalgesia, or enhanced sensitivity to pain, which has been previously reported in this model (Olechowski et al., 2009). The percentage of female and male EAE mice classified as responders in this assay is 85% and 68%, respectively (Fig 1Ci). However, only female EAE mice demonstrated lateralized behaviour (Fig 1D), meaning nociceptive responses were observed on one of two hind paws only. A chi-square test was performed and a statistically significant relationship was found between sex and laterality ($X^2(1) = 9.561$, P = 0.002; Fig 1D).

The acetone test was used to determine hypersensitivity to cold temperature using a stimulus (drop of acetone on the hind paw) that is not normally considered noxious. EAE mice exhibited a significant increase in time spent responding to the drop of acetone compared to baseline and CFA scores in both females (two-tailed t-test, $t_{16} = -2.722$, P = 0.015; Fig 1Bi) and males (two-tailed t-test, $t_{18} = -4.466$, P = <0.001; Fig 1Bii). Baseline scores between CFA and EAE mice prior to induction did not differ significantly in females (P = 0.797) or males (P = 0.195). The increase in observed attending behaviours to the hind limb applied with stimulus suggests that EAE mice exhibit allodynia, or pain in response to stimuli which are not

normally considered painful. The percentage of female and male EAE mice classified as responders in this assay is 62% and 64%, respectively (Fig 1Cii).

Motor function observed using the rotarod assay at disease onset in EAE was not significantly impaired compared to CFA females ($P = 0.195$) or males ($P = 0.841$) (data not shown). Baseline motor function scores of EAE and CFA mice prior to induction were also not different between females ($P = 0.118$) and males ($P = 0.488$). At onset of disease, the movement of hind limbs is not typically affected and therefore does not impede the ability of mice to remain on the rotarod.

3.4 Estrus Cycle Monitoring. The relationship between estrus cycle and pain behaviour was briefly assessed. At day of disease onset, vaginal lavages were performed during sedation of mice. The next day, following cresyl violet staining and cytological assessment, each female was determined to be in either proestrus, estrus, metestrus or diestrus phase of the estrus cycle. Mice were pooled from both experimental cohorts and organized by CFA, EAE or all animals as depicted in Figure 1E. No significant relationship was found between estrus phase and von Frey scores (Kruskal-Wallis H test, $H_3 = 3.181$, $P = 0.365$; Fig 1E).

3.5 Immunohistochemical Analysis. Immunohistochemical techniques were used in this study to compare nociceptive behaviour to markers of immune system function and axonal integrity. All of the following immunohistochemical markers used were assessed within the region of interest of this study, being the dorsal horn of the lumbar enlargement. The primary focus within this region is lamina 4-5 where WDR neurons reside, which is referred to as deep dorsal horn in this study. When appropriate, the superficial (lamina 1-3) dorsal horn as well as surrounding white matter was also assessed.

3.5-1 Early EAE is Associated with Spinal Cord Inflammation and Gliosis. Markers of inflammation and gliosis in the spinal cord were used to observe whether inflammatory processes at onset of disease are different between sexes. The number of CD4-positive cells in the superficial dorsal horn significantly increased with the onset of EAE compared to control in both tactile hypersensitive females and males (two-way ANOVA, disease main effect $F_{1,23} = 21.375$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 3.652$, $P = 0.017$; and males, $q = 5.574$, $P = <0.001$; no sex main effect $F_{1,23} = 1.460$, $P = 0.239$; no interaction between sex and disease $F_{1,23} = 1.023$, $P = 0.322$; Fig 2A-C). Few, if any, CD4-positive cells were observed in the dorsal horn of CFA animals. Similar to T cell influx, a significant increase in Iba1 staining with EAE responders in both the superficial (two-way ANOVA, disease main effect $F_{1,24} = 17.984$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 4.271$, $P = 0.006$; and males, $q = 4.213$, $P = 0.007$; no sex main effect $F_{1,24} = 0.000784$, $P = 0.978$; no interaction between sex and disease $F_{1,24} = 0.00312$, $P = 0.956$; Fig 3A-C) and deep dorsal horn (two-way ANOVA, disease main effect $F_{1,24} = 14.342$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 3.135$, $P = 0.037$; and males, $q = 4.415$, $P = 0.005$; no sex main effect $F_{1,24} = 0.284$, $P = 0.599$; no interaction between sex and disease, $F_{1,24} = 0.512$, $P = 0.481$; Fig 3D-F) was observed. There is no apparent sex difference in either T cell or microglial activity indicated by CD4 and Iba1 staining. An increase in GFAP-positive staining with the onset of EAE was also observed in von Frey-responding animals (Fig 4A,B,D,E). However, this effect was significantly more pronounced in males in both the superficial (two-way ANOVA, main effect of disease $F_{1,24} = 35.536$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 4.517$, $P = 0.004$ and males,

q = 7.351, P = <0.001; main effect of sex $F_{1,24}$ = 10.671, P = 0.003 followed by Tukey's method post hoc analysis for sex within EAE q = 5.659, P = <0.001 and CFA q = 1.530, P = 0.290; no interaction between sex and disease, $F_{1,24}$ = 2.357, P = 0.138; Fig 4C) and deep (two-way ANOVA, main effect of disease $F_{1,24}$ = 5.732, P = 0.025 followed by Tukey's method post hoc analysis for disease within females, q = 1.047, P = 0.466 and males, q = 3.690, P = 0.016; main effect of sex $F_{1,24}$ = 6.482, P = 0.018 followed by Tukey's method post hoc analysis for sex within EAE, q = 4.613, P = 0.003 and CFA q = 1.041, P = 0.469; no interaction between sex and disease, $F_{1,24}$ = 1.873, P = 0.184; Fig 4F) dorsal horn. Taken together, these results suggest EAE onset in both sexes is associated with changes in glial and immune cell reactivity within the dorsal horn but males exhibit an exaggerated astrocyte response to the disease.

3.5-2 Demyelination and Axon Injury is More Prominent in Male EAE Mice. Markers of axonal damage were used to examine overall axonal integrity at onset of disease compared between sexes. Fluoromyelin staining was used to quantify white matter lesions at onset of disease. Lumbar spinal cord cross sections were divided into four anatomical regions for analysis of lesions: dorsal, dorsolateral, lateral and ventral. Both female and male EAE responder mice exhibited similar numbers of white matter lesions overall (P = 0.443; Fig 5C). The percent demyelination of the cross sectional area of white matter is not significantly different between the sexes (P = 0.120; Fig 5C). White matter lesion distribution is qualitatively described in Figure 5D, where lesions are similarly distributed between sexes, the highest in lateral areas. This distribution is similarly used to describe areas of APP staining in Figure 6. APP staining was assessed as a marker of axonal damage, where presence of staining indicates aggregation of APP within axons. As the presence of APP

staining represents such a small amount of white matter area, "pockets" of staining were assessed in areas similarly used in Fluoromyelin assessment: dorsal, dorsolateral, lateral and ventral. Pockets of staining were considered regions of distinct staining accompanied by accumulating nuclei, which is similarly used to identify areas of demyelination in myelin staining. Interestingly, 100% of tissue sections analyzed from male EAE mice exhibiting tactile hypersensitivity contained injury associated, APP-positive staining. Chi-squared analysis revealed that this pattern of staining was statistically different from female EAE mice counterparts, where only 60% of tissue sections analyzed showed APP-positive staining ($X^2(1) = 4.114$, $P = 0.0425$; Figure 6C). The distribution of staining is qualitatively described in Figure 6D, where ventral areas of the spinal cord white matter appear to have the most frequent instances of APP-positive staining. As a further measure of injury and neurodegeneration, non-phosphorylated neurofilaments via SMI-32-positive cells were manually counted in both the superficial and deep dorsal horn of the spinal cord (Bernardes et al., 2016). A significant interaction was observed between sex and disease in both the superficial (two-way ANOVA, interaction $F_{1,23} = 11.973$, $P = 0.002$; main effect of disease $F_{1,23} = 31.818$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 2.205$, $P = 0.133$ and males, $q = 9.003$, $P = <0.001$; main effect of sex $F_{1,23} = 9.008$, $P = 0.006$ followed by Tukey's method post hoc analysis for sex within EAE tactile responders $q = 7.499$, $P = <0.001$ and CFA $q = 0.409$, $P = 0.775$; Fig 7A-C) and deep dorsal horn (two-way ANOVA, interaction $F_{1,23} = 6.715$, $P = 0.016$; main effect of disease $F_{1,23} = 44.195$, $P = <0.001$ followed by Tukey's method post hoc analysis for disease within females, $q = 4.102$, $P = 0.008$; and males, $q = 9.140$, $P = <0.001$; main effect of sex $F_{1,23} = 9.201$, $P = 0.006$ followed by Tukey's method post hoc analysis for sex within EAE tactile responders, q

= 6.528, P = <0.001 and CFA q = 0.394, P = 0.783; Fig 7D-F). These results indicate that SMI-32 staining is significantly pronounced in EAE male mice.

3.5-3 Spinal Innervation and Extracellular Matrix Integrity at EAE Onset. 5HT-positive staining in the deep dorsal horn was found to significantly decrease with EAE onset in both sexes (two-way ANOVA, main effect of disease $F_{1,22} = 9.015$, P = 0.007 followed by Tukey's method post hoc analysis for disease within females, q = 2.855, p = 0.047; and males, q = 3.532, P = 0.021; no main effect of sex $F_{1,22} = 3.189$, P = 0.088; no interaction between sex and disease $F_{1,22} = 0.348$, P = 0.561; Fig 8E). No apparent sex or disease effects were found in VGlut1+ staining (two-way ANOVA, no main effect of disease $F_{1,24} = 2.444$, P = 0.131; no main effect of sex $F_{1,24} = 0.144$, P = 0.707; no interaction between sex and disease $F_{1,24} = 1.200$, P = 0.284; Fig 9). Extracellular matrix integrity was examined using WFA staining. WFA is commonly used to label perineuronal nets, which are aggregates of extracellular matrix molecules (Härtig et al., 1992). Males exhibited a reduced mean brightness of WFA staining, regardless of disease state (two-way ANOVA, main effect of sex $F_{1,27} = 12.622$, P = 0.001 followed by Tukey's method post hoc analysis for sex within EAE q = 4.954, P = 0.002 and CFA q = 2.671, P = 0.070; no main effect of disease $F_{1,27} = 2.072$, P = 0.162; no interaction between disease and sex $F_{1,27} = 0.206$, P = 0.653; Fig 10E).

3.6 Golgi-Cox Staining Analysis. Golgi-Cox staining methods were used to examine dendritic morphology of deep dorsal horn neurons. Plasticity in this study is defined as a difference, either an increase or decrease, in dendritic arbourization or spine density when compared to morphologically similar cells and areas of similar cell density in CFA control animals. All cells were selected within the region of interest (lamina IV-V) and there were no statistical differences between soma diameter of cells analyzed (two-way ANOVA, no main

effect of disease $F_{1,25} = 1.857$, $P = 0.185$; no main effect of sex $F_{1,25} = 0.483$, $P = 0.493$; no interaction between disease and sex $F_{1,25} = 0.0764$, $P = 0.784$). Cells included for analysis were not obstructed such that branch paths were indistinguishable from other cells. Only dendritic spines with clearly identifiable necks were counted for spine density measures.

3.6-1 Neuronal Plasticity in Deep Spinal Cord Laminae in Male EAE. Dendritic branching analysis was used in this study to identify differences in dendritic arbourization. More specifically, branching complexity was defined as a change in the mean length of branches analyzed. Sholl analysis was also performed as part of a dendritic complexity measure. While the former branching analysis provides an estimate of gross morphological dendritic branch differences, it lacks specificity in how the pattern of branching is achieved. Sholl analysis allows one to observe how far away from the cell soma dendritic branching is occurring. Analysis of dendritic branching reconstructions (Fig 11A) revealed a significantly increased average branch length in male EAE mice exhibiting tactile pain (two-way ANOVA, sex main effect $F_{1,25} = 8.856$, $P = 0.006$ followed by Tukey's method post hoc analysis for sex within EAE, $q = 3.986$, $P = 0.009$ and CFA $q = 1.787$, $P = 0.218$; trend toward disease main effect $F_{1,25} = 4.118$, $P = 0.053$ followed by Tukey's method post hoc analysis for disease within males, $q = 3.200$, $P = 0.033$; no interaction between sex and disease $F_{1,25} = 1.854$, $P = 0.185$; Fig 11B). This branching effect was not observed in female EAE ($q = 0.713$, $P = 0.619$). Furthermore, as an exploratory sub-group analysis, male EAE responder and non-responder mice within the mechanical sensitivity assay were compared and found to statistically differ in their average branch length (two-tailed t-test, $t_8 = 3.055$, $P = 0.0157$; Fig 11C). More specifically, male EAE mice that do not exhibit tactile pain behaviour appear to have significantly shorter mean branch lengths. However, there were no

differences in Sholl analysis of dendritic branching in either sex or disease state (two-way multiple comparisons ANOVA, no main effect of disease in females $F_{1,112} = 0.0787$, $P = 0.780$; no interaction between disease and distance from soma in females $F_{7,112} = 0.350$, $P = 0.929$; Fig 12B, no main effect of disease in males $F_{1,88} = 0.0654$ $P = 0.799$; no interaction between disease and distance from soma in males $F_{7,88} = 0.278$, $P = 0.961$; Fig 12C). Moreover, when analyzing male subgroups as done previously, no effect was observed (two-way multiple comparisons ANOVA, no main effect of tactile behaviour in males $F_{1,56} = 0.0269$, $P = 0.870$; no interaction between behaviour and distance from soma in males $F_{7,56} = 0.444$, $P = 0.870$; Fig 12D).

Dendritic spines are highly dynamic structures that provide insight on neuronal activity, signaling and connectivity. In this study, dendritic spine density on deep dorsal horn neurons was assessed from primary, secondary, tertiary, quaternary and total branches. Although no difference was observed in overall spine density between sex or disease state (two-way ANOVA, no interaction between sex and disease in total branches, $F_{1,31} = 0.591$, $P = 0.448$; tertiary branches, $F_{1,31} = 0.0497$, $P = 0.825$; or quaternary branches, $F_{1,31} = 0.681$, $P = 0.417$; Fig 13A-C), a sub-group analyses of male EAE mice (tactile responders versus non-responders) reveals a significant reduction in spine density on tertiary (two-tailed t-test, $t_8 = 2.82$, $P = 0.022$; Fig 13D) and quaternary (two-tailed t-test, $t_8 = 4.293$, $P = 0.003$; Fig 13E) branches in male non-responders.

3.7 Figures and Tables

Table 1. Behavioural data summary.

		Total (n)	Cohort 1 (n)	Cohort 2 (n)	Responders (1,2*)	Laterality (1,2*)
Female	CFA	15	10	5	–	–
	EAE	20	8	12	7,10	3,5
Male	CFA	15	10	5	–	–
	EAE	19	10	9	5,8	0,0

*1,2 refers to number of animals from Cohorts 1 and 2, respectively.

Responders expressed per animal (at least one paw exhibiting hypersensitivity).

Table 1. Number of animals in each experiment and percentage of mice that are considered responders as well as displayed laterality in von Frey behavior.

Table 2. Disease parameters in female and male mice.

		Days to onset	Onset weight	Mice excluded *	Number of mortalities
Female	CFA	–	22.9 ± 0.8	–	0
	EAE	12.8 ± 3.1	19.1 ± 2.0	4 (16%)	1 (4%)
Male	CFA	–	24.3 ± 1.3	–	0
	EAE	11.1 ± 2.8	21.3 ± 1.8	0	1 (5%)

Values are mean ± standard deviation.

* due to failure to reach clinical grade 1.

Table 2. Disease parameters in female and male mice. Onset weight refers to weight of mice on day of euthanasia.

Figure 1

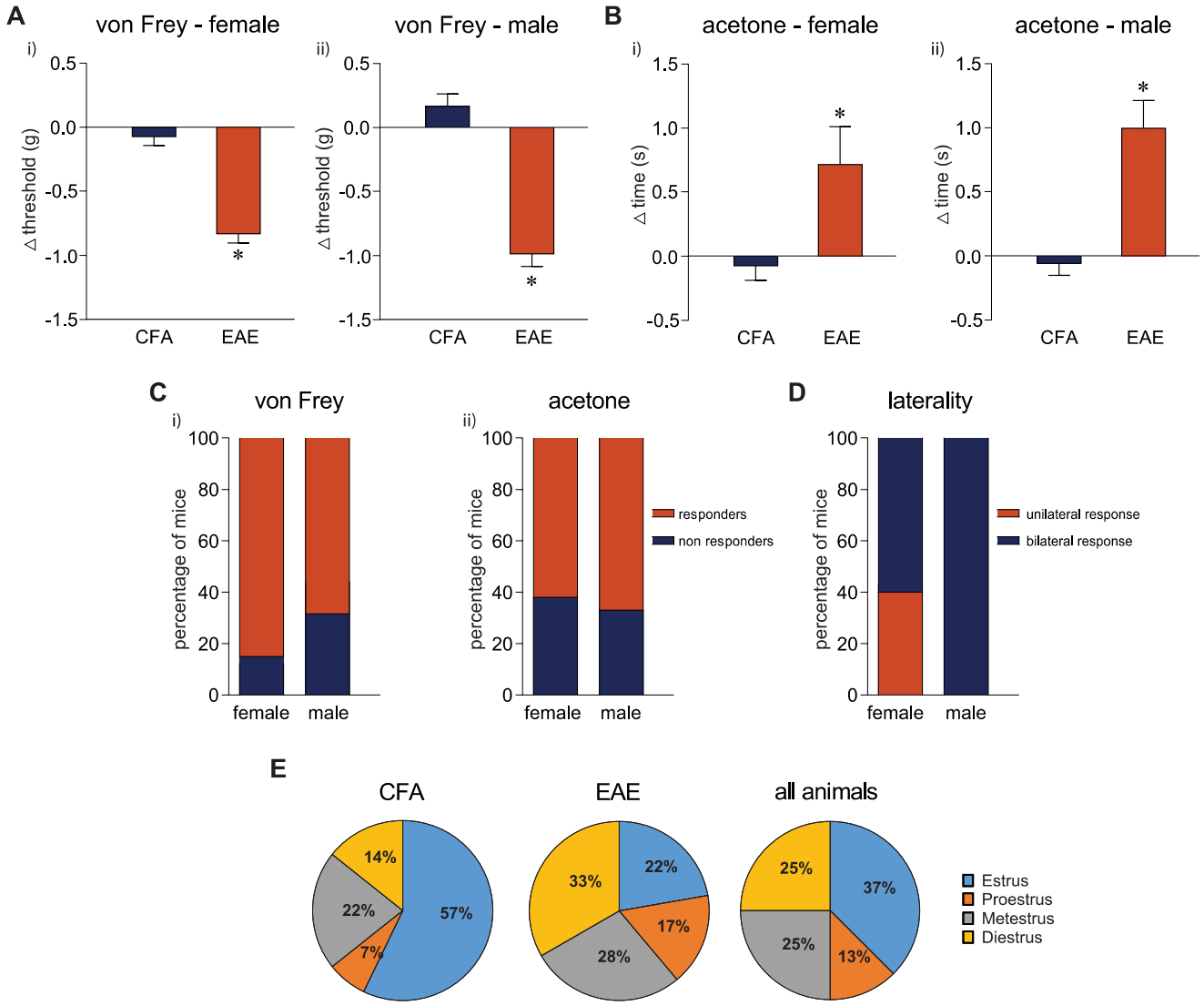

Figure 1. ***Tactile and temperature sensitivity behavioural data and estrus cycle monitoring.*** CFA (navy blue bars, A-B) represents control mice that received CFA and pertussis toxin injections only, without MOG_{35-55} peptide. EAE (red bars, A-B) represents all EAE mice tested on day of onset of disease, indicated by flaccid tail. Responders and non-responders (red and navy blue bars, respectively in C) represent percentage of EAE mice that display either a respective increase or no change in nociceptive behaviour compared to baseline scores. Unilateral and bilateral responses (red and navy blue bars, respectively in D) describe whether or not mice displayed tactile hypersensitivity in only one or both hind paws. A) von Frey monofilament behavioural assay performed in i) female and ii) male mice. Both sexes of EAE mice displayed significantly reduced thresholds to elicit nociceptive behaviours compared to CFA, both EAE and CFA values expressed relative to baseline scores. B) Acetone testing performed in i) female and ii) male mice. Both sexes of EAE mice spent a significantly increased

amount of time displaying nociceptive behaviours compared to CFA, relative to baseline scores. C) Percentage of EAE animals displaying increased nociceptive behaviours in response to i) von Frey and ii) acetone testing. Responder animals only were considered for all subsequent analysis. D) Percentage of EAE mice displaying either unilateral or bilateral nociceptive behavior in response to von Frey testing. E) Percentage of female mice in each estrus cycle phase at time of euthanasia, used to compare to von Frey scores. *P<0.05, t-test. Graphs are represented as mean ± SEM. CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis.

Figure 2

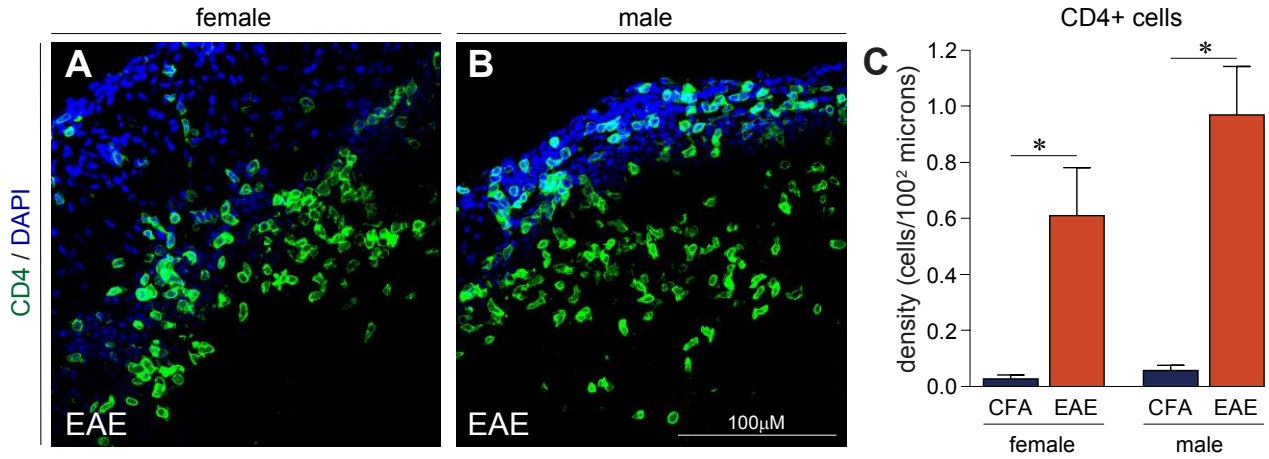

Figure 2. ***T cell influx in the superficial dorsal horn.*** CD4+ cells infiltrating the dorsal horn through the dorsal root entry zone in A) female and B) male EAE tactile hypersensitive mice. C) Density of infiltrating CD4+ cells within the superficial dorsal horn. Female and male EAE mice have a significantly increased density of CD4+ cells compared to CFA mice. Female and male groups are not significantly different from each other overall, nor are they different when compared within CFA and EAE groups. Nuclei are stained with DAPI (blue). Scale bar = 100μm in B applies to both images. *P<0.05, two-way ANOVA followed by Tukey test post hoc analysis. All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; CD4, cluster of differentiation 4.

Figure 3

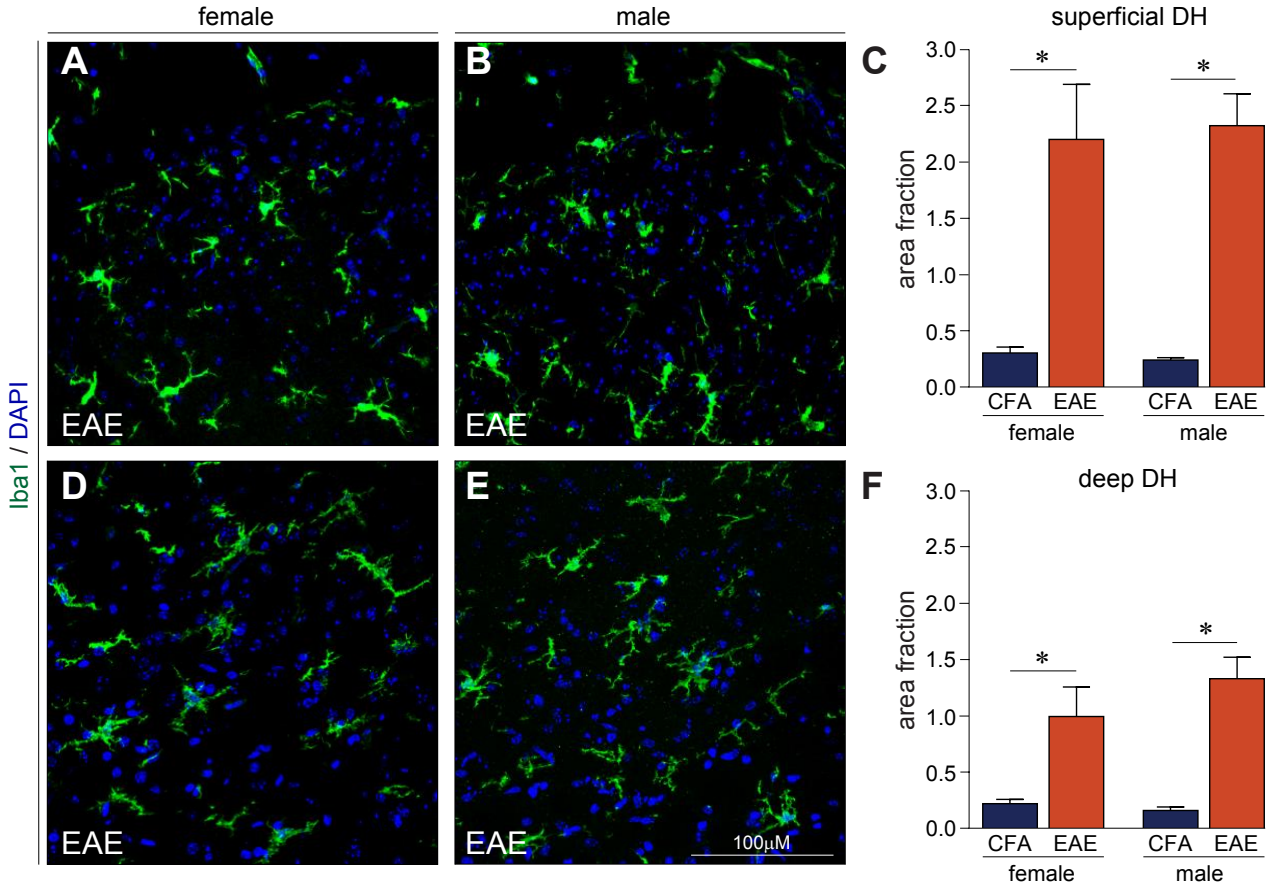

Figure 3. ***Microglial reactivity in the dorsal horn.*** Iba1+ staining in the superficial (A-C) and deep (D-F) dorsal horn, compared between female (A, D) and male (B, E) mice. C,F) Area fraction of staining within both the superficial and deep dorsal horn is significantly increased in EAE tactile responder mice compared to CFA. Female and male groups are not significantly different from each other overall, both within CFA and EAE groups. Nuclei are stained with DAPI (blue). Scale bar = 100μm in E applies to all images. *P<0.05, two-way ANOVA followed by Tukey test post hoc analysis. All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; Iba1, ionized calcium-binding adapter molecule.

Figure 4

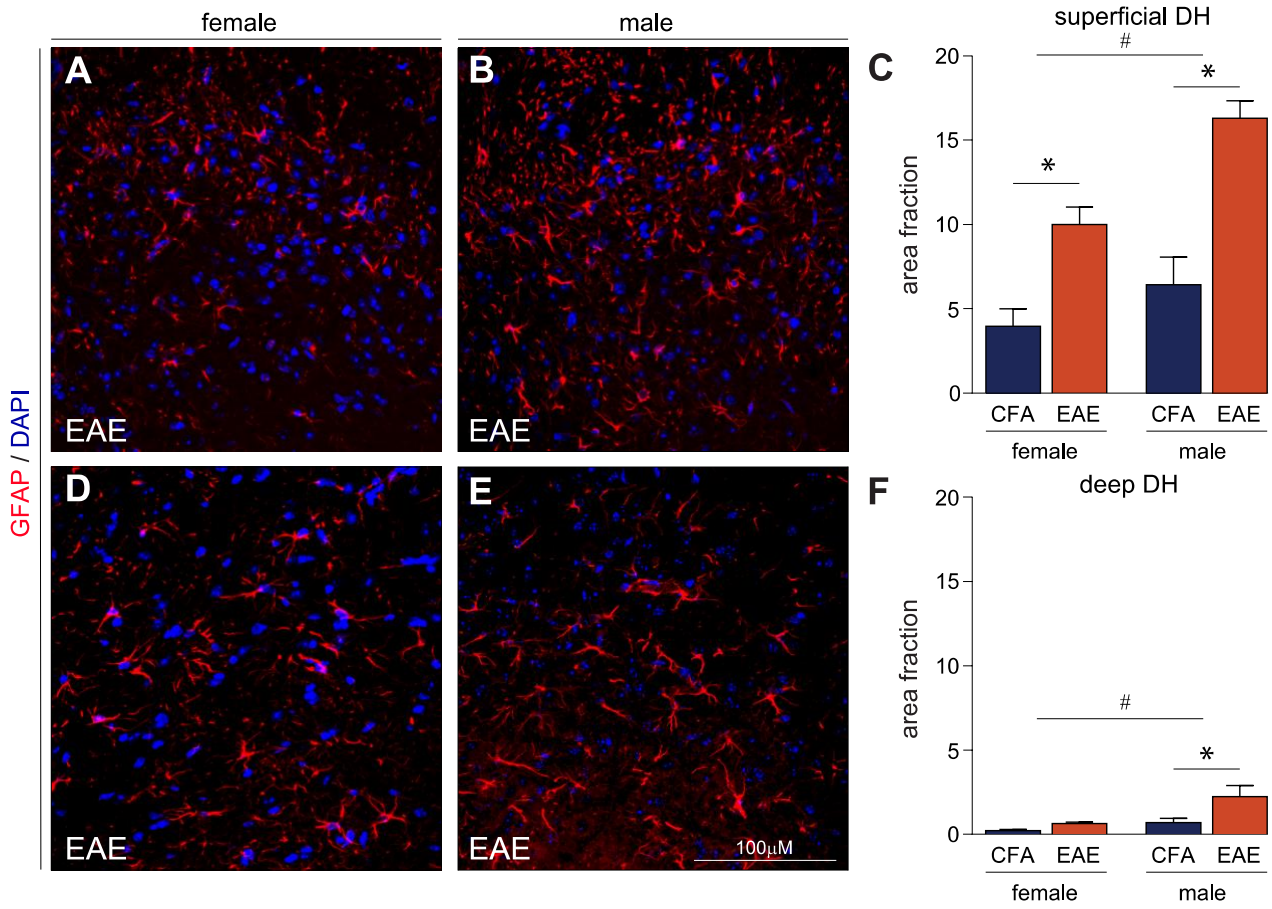

Figure 4. ***Astrocytic reactivity in the dorsal horn.*** GFAP+ staining present in both the superficial (A-C) and deep (D-F) dorsal horn, compared between sexes. C,F) Area fraction of staining within both the superficial and deep dorsal horn is significantly increased in EAE mice exhibiting mechanical hypersensitivity compared to CFA. Sex interacting with EAE is statistically significant, male EAE groups having increased area fraction in both superficial and deep dorsal horn. Nuclei are stained with DAPI (blue). Scale bar = 100μm in E applies to all images. *(disease)#(sex), $P<0.05$, two-way ANOVA followed by Tukey test post hoc analysis. All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; GFAP, glial fibrillary acidic protein.

Figure 5

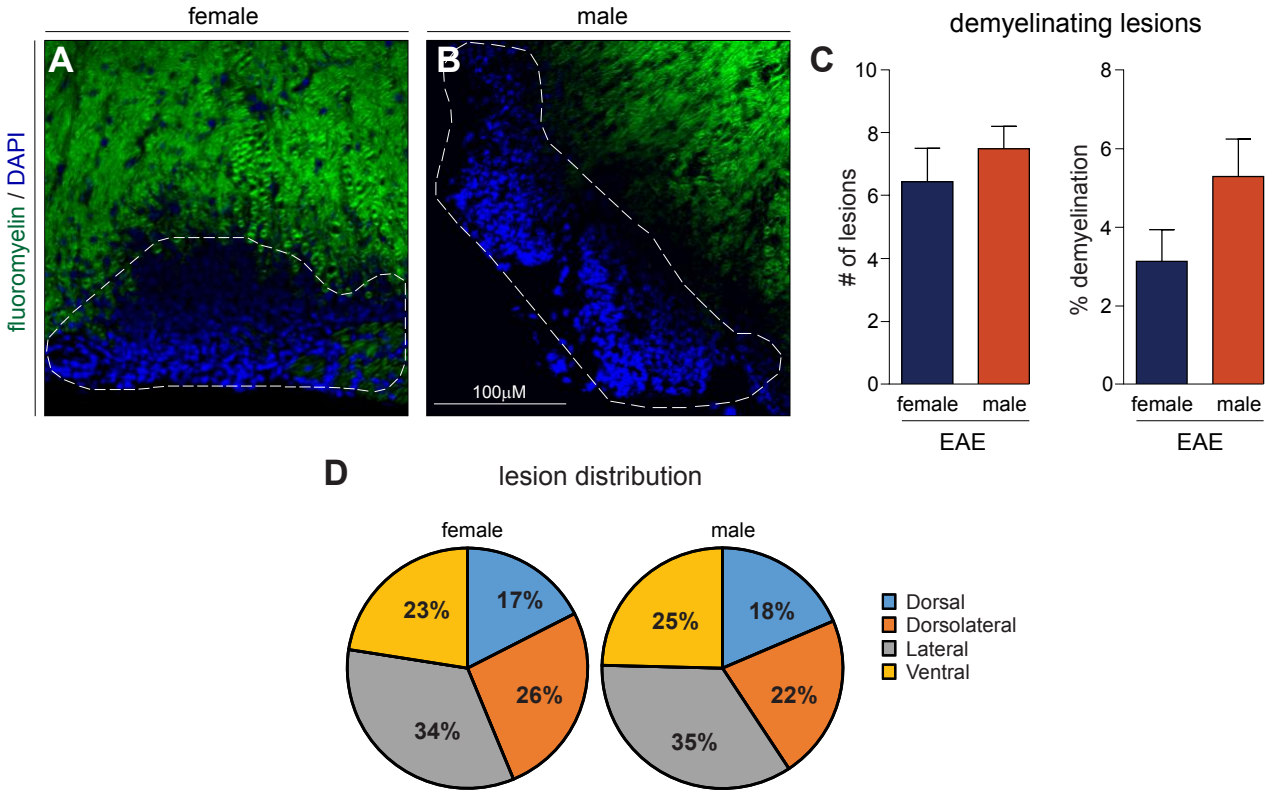

Figure 5. ***Demyelinated lesions in EAE.*** Alexa 488-conjugated Fluoromyelin staining in the lumbar enlargement used to selectively label myelin. Outlined portions of images indicate ventral lesions in A) female and B) male EAE tactile responder mice. C) Number of lesions and percent demyelination within the white matter of spinal cord cross sections are not significantly different between female and male EAE mice (student's t-test). D) Qualitative Fluoromyelin lesion distribution in the spinal cord, divided into dorsal, dorsolateral, lateral and ventral regions. Nuclei are stained with DAPI (blue). Scale bar = 100μm in B applies to both images. All graphs are mean ± SEM. EAE, experimental autoimmune encephalomyelitis.

Figure 6

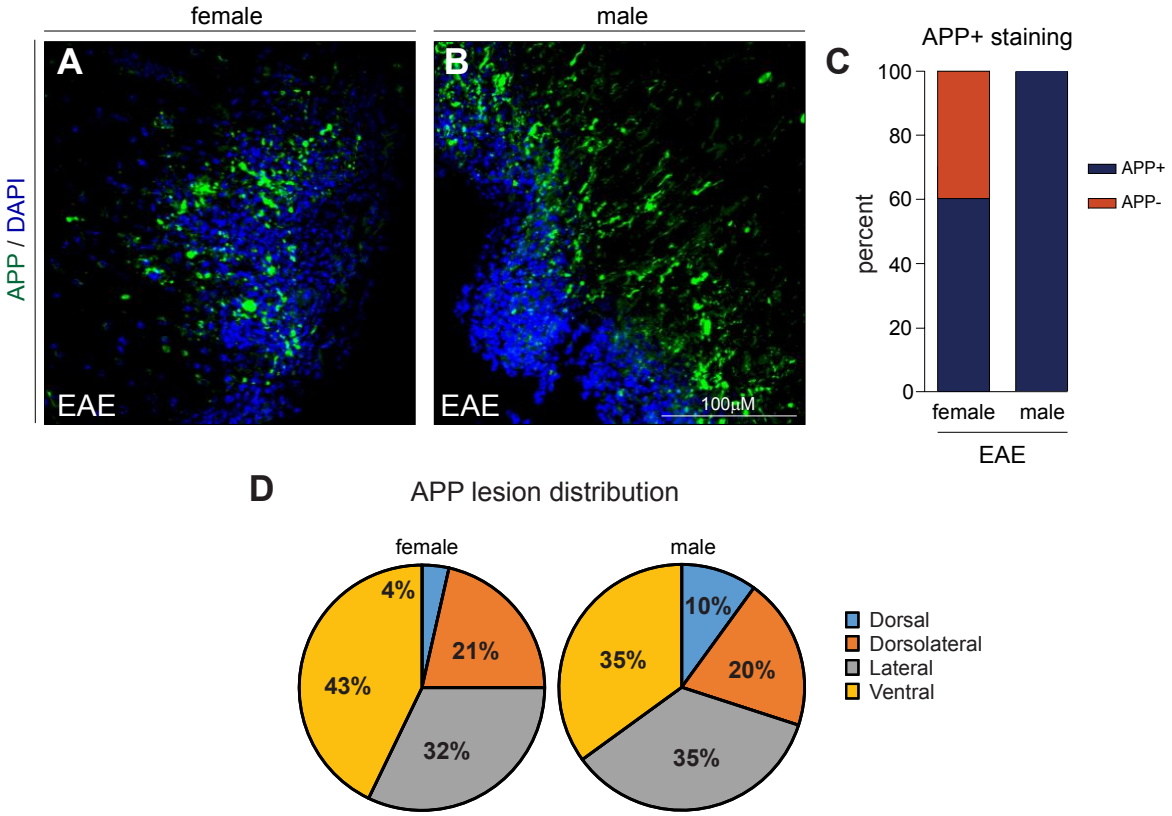

Figure 6. ***Axonal damage in spinal cord white matter.*** APP+ staining present in lateral regions of the white matter in both A) female and B) male EAE tactile responder mice. C) Percentage of mice with APP+ staining present in EAE responder animals. Presence of absence of APP+ staining is significantly different between females and males. D) Qualitative APP+ staining distribution in white matter divided into dorsal, dorsolateral, lateral and ventral regions. Nuclei are stained with DAPI (blue). Scale bar = 100μm in B applies to both images. Significance set at $P<0.05$, Chi-squared test. EAE, experimental autoimmune encephalomyelitis; APP, amyloid precursor protein.

Figure 7

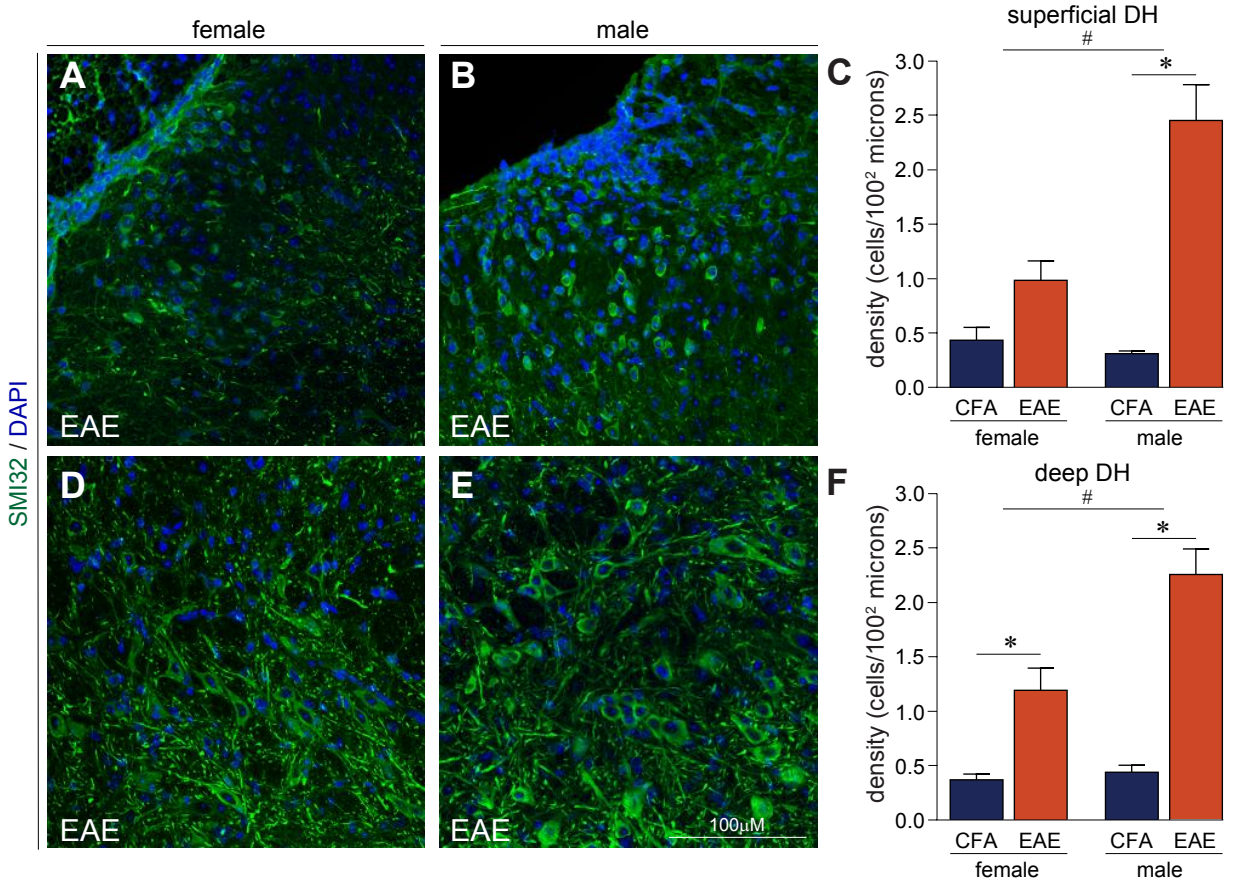

Figure 7. ***Neurodegeneration in the dorsal horn.*** SMI-32+ cells in the superficial (A-C) and deep (D-F) dorsal horn, compared between female (A,D) and male (B,E) mice. C) Density of SMI-32+ cells in the superficial dorsal horn is significantly increased in male EAE mice only. Male mice overall are significantly increased compared to females. F) Density of SMI-32+ cells in the deep dorsal horn is significantly increased in female and male EAE mice. In tactile hypersensitive EAE, male mice have an increased presence of SM-I32+ cells compared to female mice. Nuclei are stained with DAPI (blue). Scale bar = 100μm in E applies to all images. *(disease)#(sex), $P<0.05$, two-way ANOVA followed by Tukey test post hoc analysis. All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; SMI-32, neurofilament H nonphosphorylated.

Figure 8

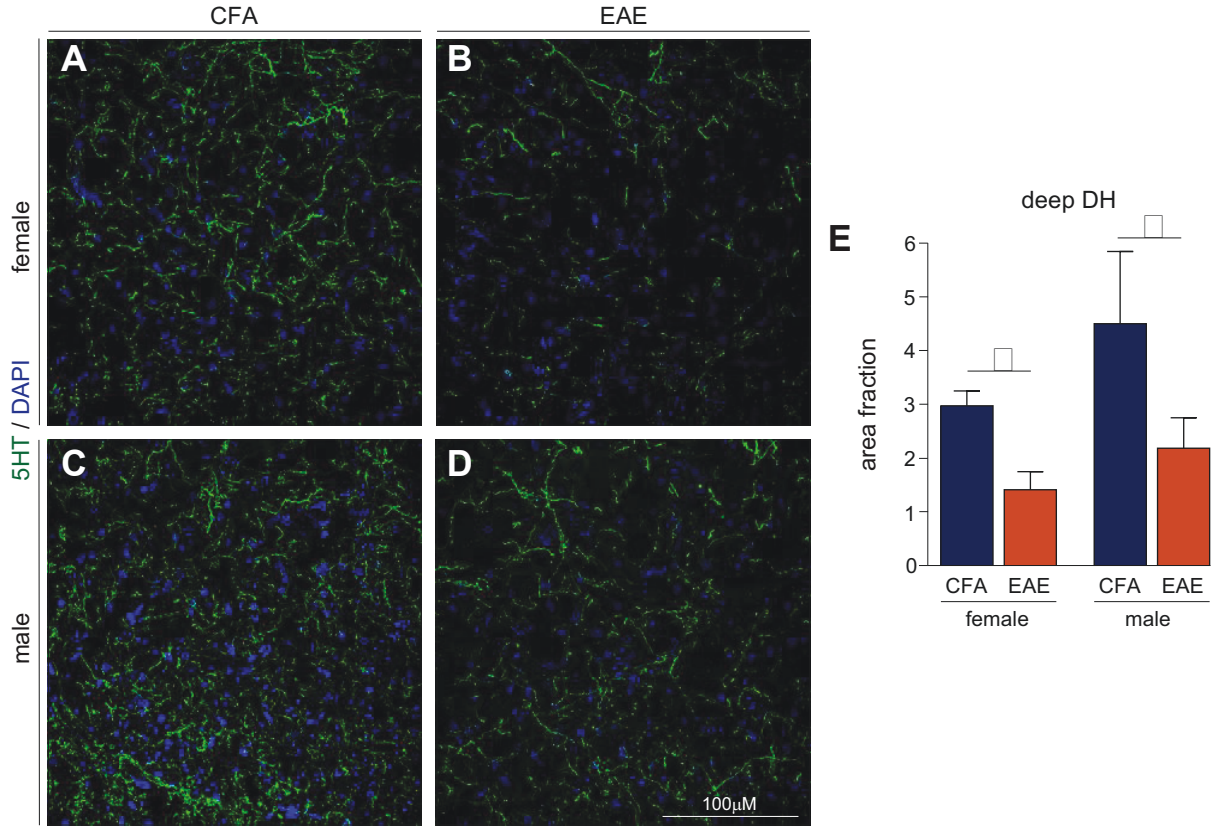

Figure 8. ***Serotonergic projections in the dorsal horn.*** 5HT+ staining present in the deep dorsal horn compared between female (A-B) and male (C-D) mice. E) Area fraction of staining within the deep dorsal horn is significantly reduced in at onset of EAE responder mice compared to CFA in both sexes. Nuclei are stained with DAPI (blue). Scale bar = 100μm in D applies to all images. *P<0.05, two-way ANOVA followed by Tukey test post hoc analysis. Graph is mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; 5HT, serotonin.

Figure 9

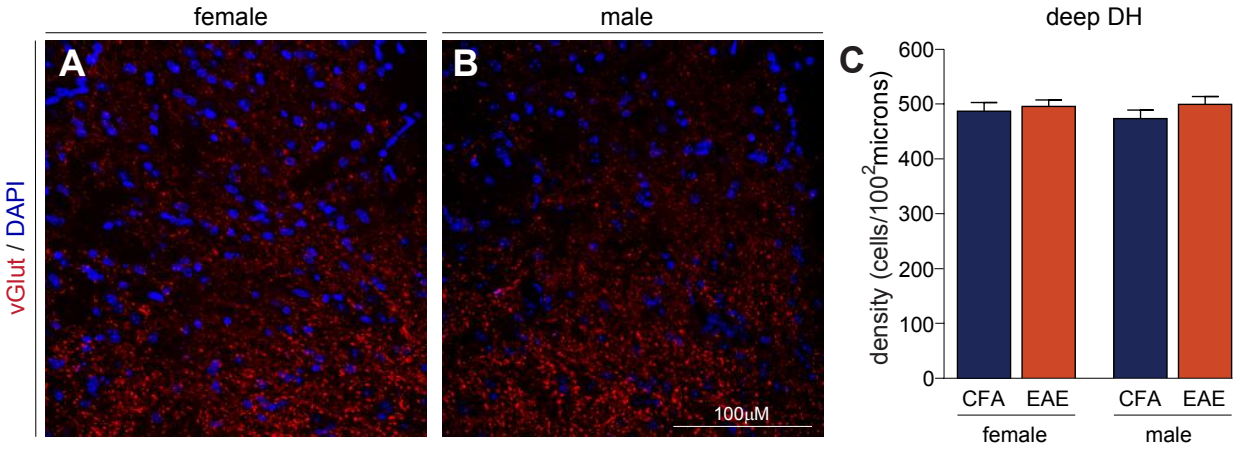

Figure 9. ***Synaptic puncta density in the dorsal horn.*** VGlut1+ staining of presynaptic puncta in the deep dorsal horn of A) female and B) male EAE tactile responder mice. C) Puncta density is not significantly different between disease or sex. Nuclei are stained with DAPI (blue). Scale bar = 100μm in B applies to all images. Significance set at $P<0.05$, two-way ANOVA followed by Tukey test post hoc analysis. Graph is mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; vGlut, glutamate vesicular transporter.

Figure 10

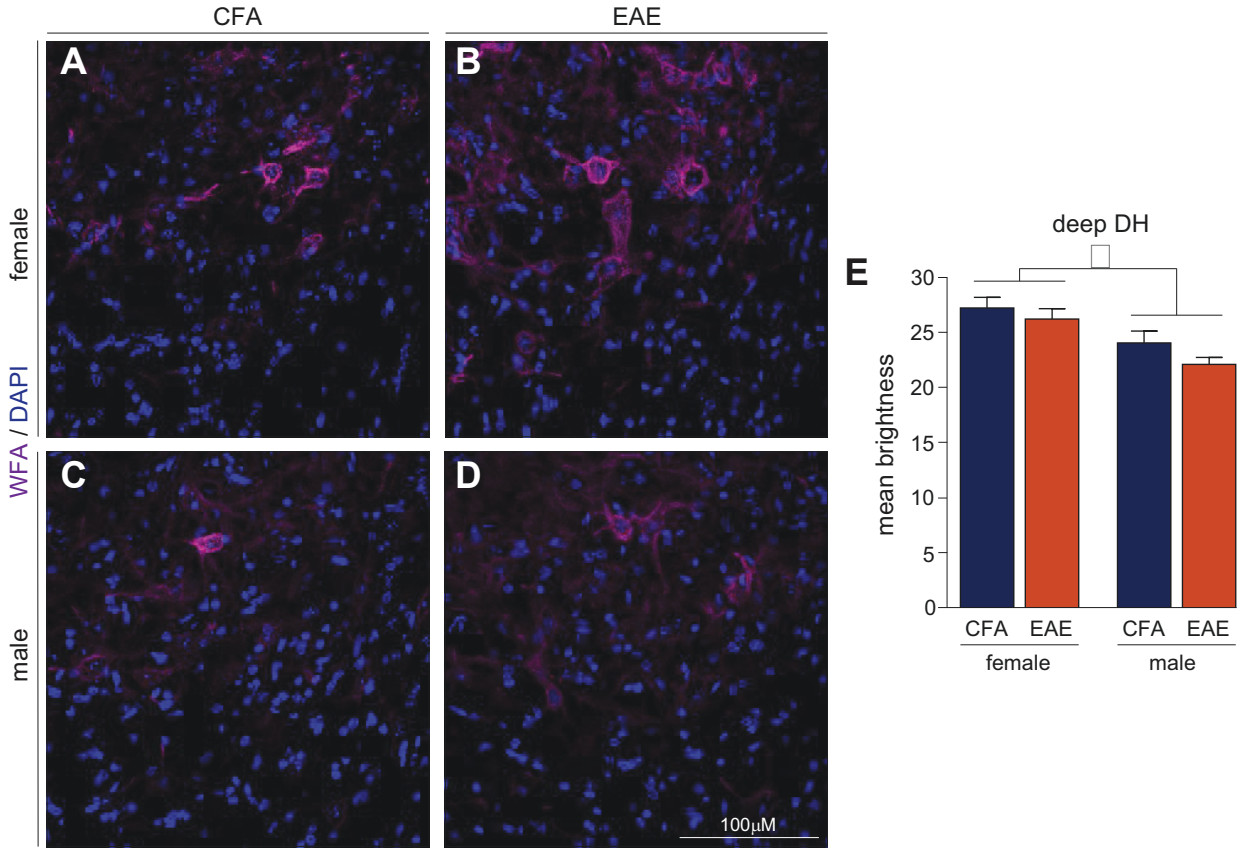

Figure 10. ***Extracellular matrix integrity in the dorsal horn.*** WFA+ staining the deep dorsal horn of female (A,B) and male (C,D) mice. E) Mean brightness of WFA+ staining is significantly reduced in male mice overall. Nuclei are stained with DAPI (blue). Scale bar = 100μm in D applies to all images. *P<0.05, two-way ANOVA followed by Tukey test post hoc analysis. Graph is mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; DH, dorsal horn; WFA, Wisteria floribunda agglutinin.

Figure 11

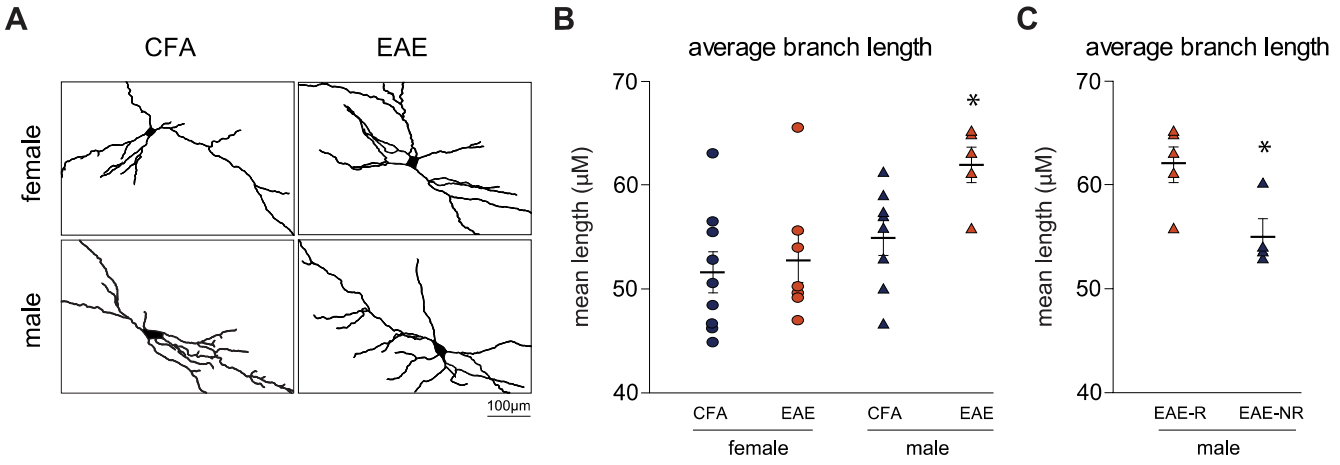

Figure 11. ***Dendritic branching of deep dorsal lamina neurons in the dorsal horn.*** A) Schematic diagram of dendritic arbour traces made from the ImageJ Simple Neurite Tracer plugin for branching analysis. B) Average branch length is significantly increased in male EAE mice showing pain behaviours. Navy blue and red points denote CFA and EAE responder animals, respectively; circles and triangles are used to represent each female and male animals, respectively. C) Exploratory sub-group analysis of male responder and non-responder EAE mice are statistically different from one another. Red and navy blue triangles represent male EAE responders and non-responders, classified by their von Frey behavioural scores. Scale bar = 100µm in A applies to all schematic images. *P<0.05, two-way ANOVA followed by Tukey test post hoc analysis (B) and t-test (C). All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EAE-R, responders; EAE-NR, non-responders.

Figure 12

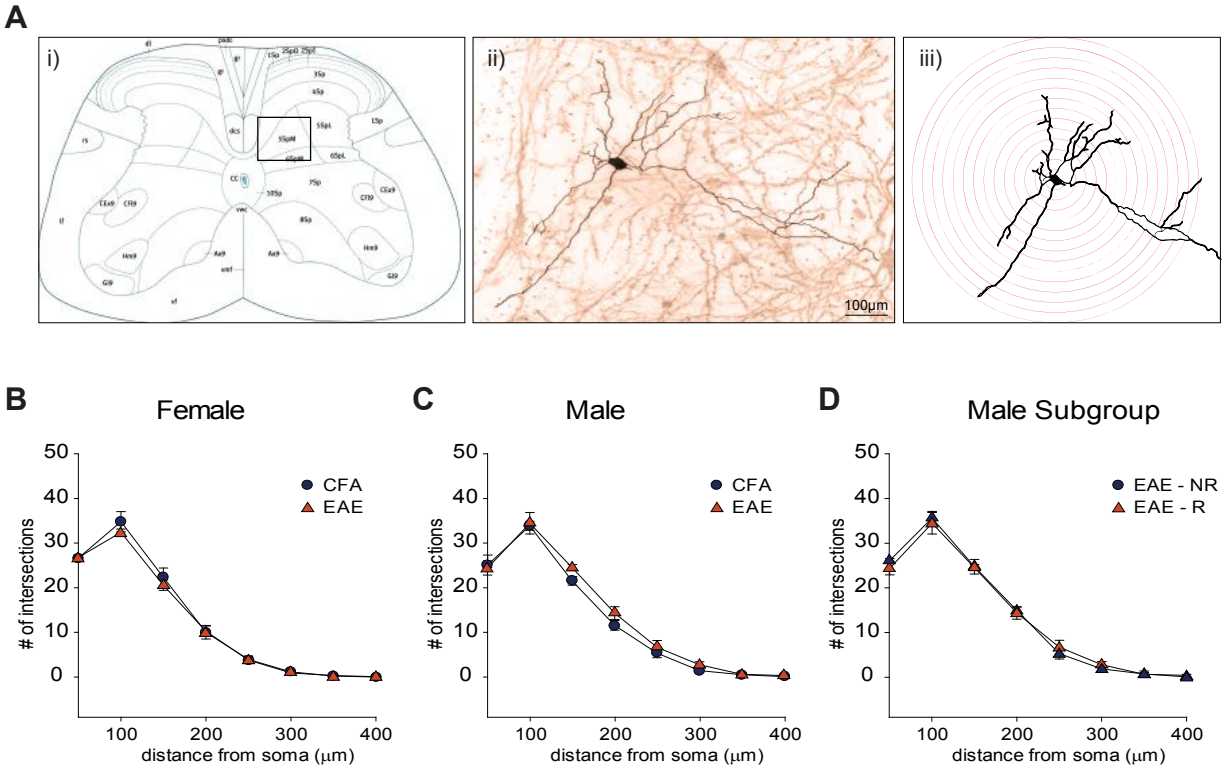

Figure 12. ***Sholl analysis of deep dorsal lamina neurons in the dorsal horn.*** A) Schematic diagram of i) location cells were chosen from and ii,iii) how cells were traced and analyzed using the ImageJ Simple Neurite Tracer plugin. B,C) There is no significant difference between female or male EAE tactile responder mice compared to respective CFA mice. Navy blue circles and red triangles represent CFA and EAE animals, respectively, at varying distances from the cell soma. D) There was no difference found within male subgroup analysis comparing EAE responders and non-responders. Navy blue circles and red triangles represent male EAE non-responders and responders, classified by their von Frey behavioural scores. Scale bar = 100µm in Aii applies to Aii image only. $P<0.05$, two-way ANOVA followed by Tukey test post hoc analysis. All graphs are mean ± SEM. ANOVA, analysis of variance; CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EAE-R, responders; EAE-NR, non-responders.

Figure 13

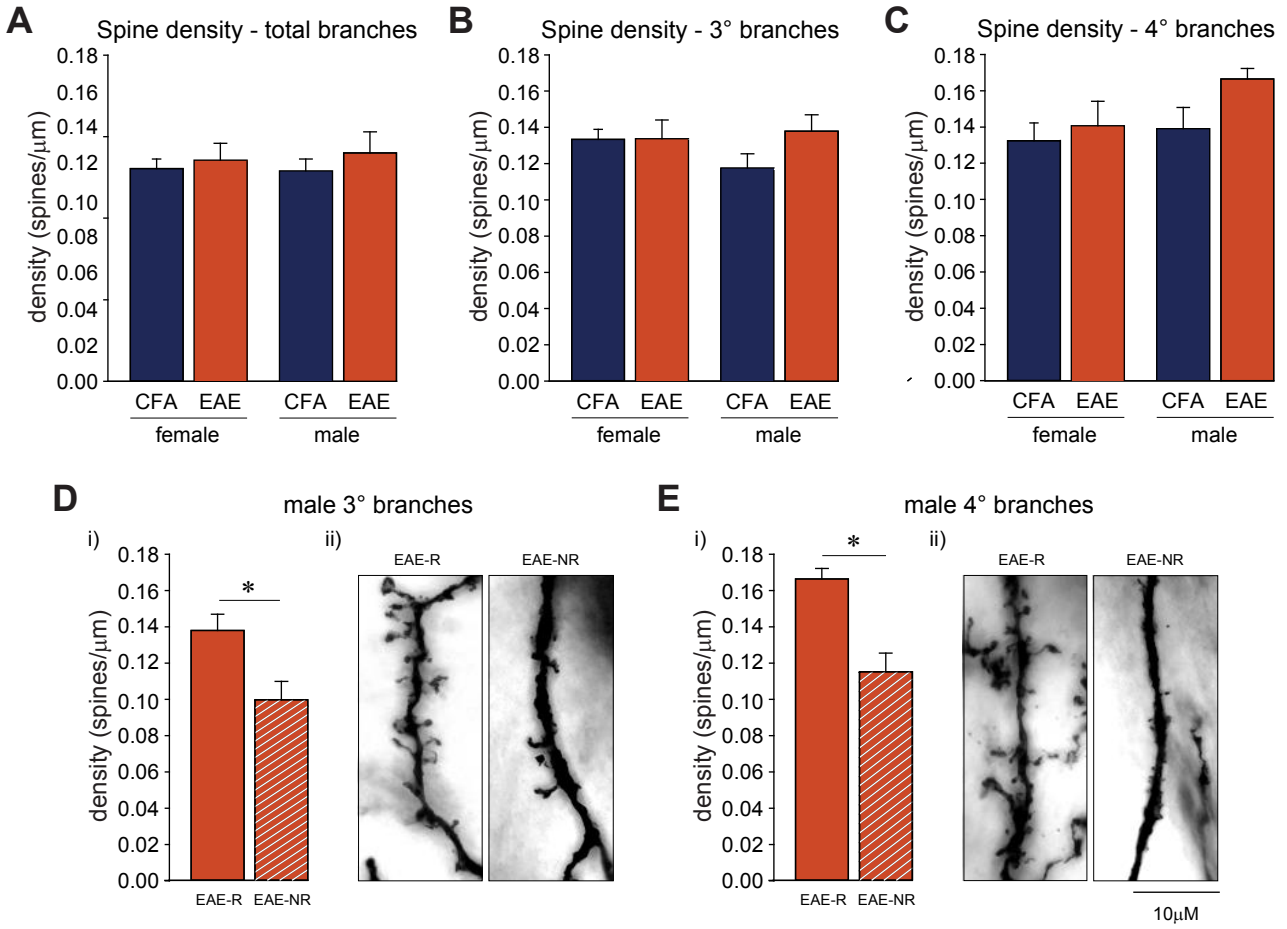

Figure 13. ***Dendritic spine density of deep dorsal lamina neurons in the dorsal horn.*** Navy blue and red bars represent CFA and EAE responder groups, respectively. There are no significant differences in spine densities of A) total, B) tertiary, and C) quaternary branches between either sex or disease state. Subgroup analysis of males into responder (solid red bars) and non-responder (striped red bars) mice revealed significant differences in D) tertiary and E) quaternary branches. Scale bar = 10µm in Eii applies to all images. *P<0.05, t-test. All graphs are mean ± SEM. CFA, Complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EAE-R, responders; EAE-NR, non-responders.

4. Discussion

4.1 The Present Study

This study investigates sex differences in inflammation, axon injury, and plasticity of early onset MOG_{35-55}-induced EAE mice displaying nociceptive behaviours. I assessed nociceptive behaviour by two modalities, mechanical and temperature hypersensitivity, and employed a classification system used to describe mice as responders or non-responders. I explored inflammatory and axon injury markers by immunohistological methods, and differences in plasticity measures by Golgi-Cox staining. In addition, this study takes into consideration the possible effect of estrus cycle on nociception as well as potential motor confounds in EAE.

4.2 Disease Course and Day to Onset

No sex difference was observed in the number of days taken to reach disease onset in this study. While both sexes of EAE mice demonstrate neurological impairments compared to CFA mice, the average day to onset observed was 12.8±3.1 and 11.1±2.8 in females and males, respectively (reported in Table 2). The time taken for male and female C57BL/6 EAE mice to reach these clinical signs has been similarly described by previous work (Papenfuss et al., 2004; Rahn et al., 2014). It is important to note that this study considered the onset time point only and cannot make conclusions of later time points where sex differences in disease presentation may occur. Furthermore, these results are specific to EAE protocols from the Kerr laboratory, which may differ from other forms or severity of EAE that use other immunization protocols, mouse strain, age or adjuvant. For example, female SJL mice demonstrate more severe neurological signs when immunized with myelin basic protein, while males display more severe neurological deficits in the Theiler's murine encephalomyelitis virus model

(Papenfuss et al., 2004; Lynch et al., 2008). It is important to consider which model of chronic inflammation and demyelinating disease will best address the research questions of interest. Sex differences in the physiological effects of various components of EAE induction, such as MOG_{35-55} and pertussis toxin, may present potential confounds (e.g. fever or change in body temperature) in disease presentation and progression (Bittner et al., 2014). Such reactions to various immunization protocols should be carefully monitored in future studies.

4.3 Behaviour

Traditionally, our lab has used the EAE model to elucidate mechanisms of pain by investigating the cellular machinery of animals demonstrating nociceptive behaviours. Previous behavioural assays used in this context by the Kerr laboratory include the von Frey monofilaments, acetone test and the Hargreaves test (Olechowski et al., 2009, 2013). In this study, I used von Frey monofilaments and the acetone test to measure two modalities of pain. In addition, I used the rotarod assay to measure gross locomotor ability and coordination throughout experiments. All testing conditions were kept under similar noise levels, room temperature, time of day and performed blind to treatment groups to minimize response variability. It is worth noting that, in future studies, the statistical analyses used in this project to compare behavioural scores to estrus cycle data as well as measures of inflammation and plasticity should be supplemented with correlations to better understand whether one factor predicts another.

Similar to humans with MS, a portion of EAE animals do not develop behavioural signs of pain hypersensitivity (Österberg et al., 2005; Rahn et al., 2014). This phenomenon was observed in both behavioural assays used in this study. These observations drove further subset analyses which attempt to describe the susceptibility of some EAE mice developing pain

behaviours while others do not. As pain symptoms in MS patients are highly variable in presentation, severity and duration, it is probable that multiple mechanisms of action are contributing to the development of pain in the disease. Perhaps, in future studies, non-responders or mice exhibiting laterality in nociceptive behaviour may serve as internal controls to delineate the changes that may underlie the development of nociception. Pathology on the basis of laterality was not specifically addressed in this study, as there were not enough animals in these groups to conduct statistical analyses. Future experiments should be designed in order to review the concept of laterality in nociception in more depth. As laterality of somatosensory processing and pain has been demonstrated in humans, namely a heightened perception of painful stimuli on the left side of the body, there is merit in investigating laterality at the level of the spinal cord corresponding to behaviour in EAE (Merskey and Watson, 1979; Coghill et al., 2001; Klemenz et al., 2009).

4.3-1 Mechanical Hypersensitivity

The von Frey monofilaments assay was used to measure mechanical allodynia at onset of EAE. In general, nociceptive thresholds were similarly reduced in both female and male EAE mice at onset of disease compared to respective CFA mice. Moreover, mice were described as either responding or non-responding as a method of characterizing the population of animals that did not display robust nociceptive behaviours at onset of disease. This classification allowed for subset analyses to be performed in an attempt to understand risk or protective factors in developing pain in EAE. Interestingly, laterality in nociceptive behavior was observed in female but not male mice. More specifically, 40% of female EAE mice demonstrating nociceptive behaviours exhibited reduced von Frey thresholds on only one paw. Male nociceptive behaviour in the von Frey testing was exclusively bilateral (observed

in both hind paws). These findings suggest that there may be a different mechanism by which mechanical pain is elicited between sexes. Furthermore, female EAE mice exhibiting lateralized behaviour may be protected either at spinal or supraspinal areas that spares their nociceptive sensitivity from being as severely affected as males. The exact mechanisms underlying this process is uncertain, though there is evidence to support maladaptive functional and structural plasticity in the primary somatosensory cortices of female EAE mice exhibiting heightened nociceptive responses to tactile stimulation (Potter et al., 2016). Perhaps, in female EAE mice that appear less sensitive to mechanical stimuli, laterality in behaviour is determined in part by top-down inhibition maintained in the somatosensory cortex. On the other hand, the behavioural findings in this study were observed strictly at the onset of clinical signs and may not reflect behaviour at a chronic time point. It is worth noting that another experiment designed to specifically address laterality in pain behaviour would be needed with the appropriate number of animals. Though this laterality phenomenon was observed consistently between two cohorts of animals, this study was not designed to investigate the cause of lateralized behaviour to tactile stimuli in female EAE mice.

4.3-2 Temperature Hypersensitivity

The acetone test was used as a measure of cold temperature allodynia at onset of EAE. Overall, both female and male EAE mice exhibited increased response times to innocuous cold stimuli compared to CFA mice. Cold allodynia is apparent early in the disease course; however, a greater percentage of mice did not exhibit nociceptive behaviour compared to the von Frey monofilaments testing. Furthermore, the laterality in behaviour was not addressed in this testing modality. While mice were also classified as responders and non-responders in this assay, further analyses were compared to tactile hypersensitivities only. This is in part

because, given the different somatosensory modalities used to assess pain in this study, there are differing modulatory pathways between tactile and temperature stimuli (Braz et al., 2014; Gangadharan and Kuner, 2015). Though laterality in nociceptive responses was also present in the acetone test, these animals did not completely coincide with animals displaying laterality in von Frey monofilaments testing. However, WDR neurons are implicated in processing both mechanical and thermal stimulation, which would be of interest to study further in future studies (Tan et al., 2008b; West et al., 2015). While temperature and tactile sensitivities both pose major sensory disturbances early in EAE progression, only one modality was included within the scope of this study. Correlating markers of inflammation and neurodegeneration to multiple sensory modalities provides an interesting direction for future research.

4.3-3 Motor Function

The Rotarod assay was used in this study to measure locomotor function and ensure that motor impairment did not impede responding to other behavioural assays. At onset of disease, neither female nor male EAE mice exhibited a significant reduction in locomotor ability compared to CFA. Partial tail paralysis occurs at onset of clinical signs, however at this point in the disease locomotion is not significantly impaired. The absence of locomotor deficits in this study suggests that a lack of response during behavioural assay testing is not due to impaired motor functioning. Rather, EAE mice that are not exhibiting nociceptive behaviour may be hyposensitive to the modalities tested in this study. The motor control assay used in this study may not completely eliminate motor confounds present, which can impair the presence of withdrawal responses in nociception assays. Given that chronic stages of EAE

present with major motor complications, it is important to closely evaluate the adequate exclusion of motor confounds in future studies.

4.4 Estrus Cycle Monitoring

Many studies have pointed to the role of sex hormones in MS and chronic pain states, as women are disproportionately affected by such conditions. Not only has proestrus phase been reported to halt progression of motor deficits in EAE, but progesterone administration prior to immunization has also demonstrated protective effects in the form of delayed disease onset and reduced neurological deficits (Garay et al., 2012; Rahn et al., 2014). Results from this study did not demonstrate any relationship between estrus cycle phase and pain behaviour or disease course. However, these observations are taken from a small number of animals and may not accurately reflect a shift in pain behaviour according to hormonal levels. In addition, it is important to consider changes in estrus cycling at chronic time points in EAE, which is often shortened and irregular (Jaini et al., 2015). In future studies, correlational statistics may be more beneficial to evaluate the relationship between estrus cycle and nociception in EAE.

4.5 Immunohistochemistry

Chronic inflammation is a hallmark feature of MS pathology. It is known that female susceptibility to MS and other autoimmune conditions is elevated, suggested in part by the female immune response (Dunn et al., 2007). An increasing body of research demonstrates differing immune cell profiles between the sexes in the periphery both prior to and following injury. It is proposed that males utilize microglial-dependent pathways for pain processing, while females likely use adaptive T lymphocytes (Sorge et al., 2015). The peripheral immune system of female mice have larger quantities of peritoneal macrophage populations, CD4+ T cells and express more chemokines in response to injury (Sorge and Totsch, 2017). Sex-

specific populations of T cells have been demonstrated, possibly making women more susceptible to certain autoimmune conditions due to having a more reactive, Th1 phenotype, in turn producing higher levels of interferon gamma (Dunn et al., 2007; Zhang et al., 2012).

Also noteworthy are the neuron-glia interactions that facilitate immune system responses through the regulation of inflammatory mediators. Males may be skewed toward a Th17 phenotype which contribute to autoimmunity by releasing pro-inflammatory cytokines that ultimately activate microglia and further perpetuate immune infiltration (Zhang et al., 2012; Moynes et al., 2014). While there were no statistically significant sex differences in either Iba1- or CD4-positive staining in this study, these markers both drastically increase with EAE. More specific microglial and T cell markers could also be explored in future studies. We did however observe increased GFAP-positive staining in male compared to female mice with EAE. Astrocyte activation has been implicated in neuropathic pain states by way of releasing pro-inflammatory cytokines which subsequently modify spinal cord NMDA synaptic signalling (Chiang et al., 2012). Furthermore, the capacity of astrocytes to remodel dendritic spines has been demonstrated in an experience-dependent manner (Perez-Alvarez et al., 2014). While the EAE non-responder sample sizes in this study are too small to make comparisons regarding how astrocytes contribute to the development of or protection from certain pain phenotypes, this is an interesting line of work to pursue. The significant increase in the amount of T cells and glia in the dorsal horn have been demonstrated previously in EAE (Olechowski et al., 2009). While a great deal of research is still being done in this area, it is worth investigating the role of sex-specific T cell and glial activation in the development and maintenance of chronic pain. The interconnected relationship between elevated pro-inflammatory cytokines and chemokines, reactive gliosis, and amplified neuronal

hyperexcitability have been largely implicated in the development and persistence of chronic pain in MS (Zhang and An, 2007; Zhuo et al., 2011). While this study does not address the various measures of inflammation in EAE non-responder mice, we suspect these animals are less severely affected and perhaps more closely reflect levels observed in CFA mice. Such a claim would need to be substantiated with future studies.

Axon injury is a prominent feature of chronic inflammation and demyelinating conditions such as MS, and is reflected in the EAE model. This notion has been reinforced via immunohistochemical staining used in this study. In the spinal cord, it is evident that EAE is associated with significant demyelination, and with similar lesion distributions between the sexes. Accompanying this myelin loss were pockets of APP-positive staining, found mostly within lateral and ventral regions of the white matter where portions of the spinothalamic tracts reside (Sengul and Watson, 2012). Both lateral and ventral spinothalamic tracts contribute to the transmission of pain, pressure and temperature sensations. Interestingly, APP staining was more prominently and consistently observed in EAE males. Amongst the many components of neurodegeneration in MS is the accumulation of APP, leading to disrupted axonal integrity and transport (Hauser and Oksenberg, 2006). Along the same lines, SMI-32 staining revealed a marked increase in non-phosphorylated neurofilaments in EAE males within the dorsal horn. Phosphorylation of neurofilament proteins is considered an important process in regulating stability of the cytoskeleton (Lee et al., 2014). Accumulation of the non-phosphorylated neurofilament occur as a result of axonal damage in demyelinating lesions (Trapp et al., 1998). The increased presence of SMI-32 positive cell bodies may suggest a more detrimental neurodegenerative phenotype in the male EAE spinal cord.

Within the region where WDR neurons reside, components of the extracellular matrix and spinal innervation markers were assessed in order to understand potential regulators of plasticity. No difference between sex or disease state was found in excitatory presynaptic terminal (VGlut1 staining) densities within the dorsal horn, which has been previously shown to increase in the primary somatosensory cortex in early stages of EAE (Potter et al., 2016). It is uncertain whether or not these changes would be reflected at the level of the spinal cord at later time points in the disease. EAE mice exhibited a reduction in serotonin-containing fibers, which complements previous findings that, while serotonin plays a role in regulation of nociception, this process may be deficient in females (Mifflin et al., 2015). Males on the other hand, regardless of disease state, displayed a global reduction in WFA-stained perineuronal net components. Early disruption of specialized extracellular matrix structures, which normally serve protective functions to associated neurons, may contribute to maladaptive structural plasticity in male EAE mice (Härtig et al., 1992). However, potential histological confounds such as cell death and edema need to be considered in the future to strengthen the findings presented in this study. Significant cell death, for example, may impact the accumulation of resident macrophages and alter the excitatory-inhibitory balance of the local environment (Labrakakis et al., 2011).

4.6 Golgi-Cox

The Golgi-Cox method of staining, characterized by high contrast intracellular staining, provides insight into neuronal morphology and microstructure (Ramon-Moliner, 1970; Koyama, 2013). While only a small percentage of cells are impregnated, this technique has been used vastly to characterize measures of plasticity such as dendritic branch and spine density (Kolb and Whishaw, 1998; Das et al., 2013). For the purpose of this study, the Golgi-Cox technique was

used to measure plasticity changes in presumptive WDR neurons at onset of clinical signs of EAE. Furthermore, an exploratory subset analysis of responding and non-responding males in EAE was performed in an attempt to understand whether deep dorsal horn neuronal plasticity is associated with tactile hypersensitivity.

While male EAE mice exhibited longer average dendritic branch length of deep dorsal horn neurons, the dichotomy between responding and non-responding males raising interesting questions for future study. Despite no differences found in Sholl analysis, the change in average branch length in the male EAE suggests a gross increase in dendritic arbourization as opposed to sprouting of specific branch types (i.e. secondary, tertiary). Furthermore, male EAE mice that did not exhibit tactile hypersensitivity have an average branch length more similar to that of CFA animals, who also do not display pain behaviours. Similar to dendritic branching results, there is an apparent contrast in spine density between male subgroups. In both tertiary and quaternary branches male EAE non-responders have a marked decrease in spine density compared to hypersensitive males. Taken together, these changes in deep dorsal horn neuron morphology suggests a plasticity-dependent modulation of pain behaviour in male and not female mice with EAE. However, it is important to consider whether cells stained by the Golgi-Cox method truly reflect the cell density in any region of interest and to control for this uncertainty. An increase or atrophy of spinal cord cell densities may influence dendritic plasticity, which is significant in order to distinguish potentially adaptive, neuroplasticity mechanisms from changes as a result of injury. This study cannot disentangle with certainty whether branching and spine density plasticity changes observed are a result of degeneration versus sprouting mechanisms, but provides interesting points for future discussion.

A direction for follow up research may measure activity-related changes of WDR neurons at EAE onset. In spinal cord injury models, dorsal horn neurons have been proposed to amplify both innocuous and noxious stimuli which relay amplified pain signaling. This exaggerated nociceptive and hyperexcitability of second-order neurons, including WDR neurons, is thought to occur in part by sodium channel dysregulation (Waxman and Hains, 2006). Moreover, the involvement of microglia and astrocytes in immune-mediated synaptic pruning is also thought to alter functional connectivity in CNS neurodegenerative diseases (Stephan et al., 2012). This idea may fit with results from this study of elevated GFAP staining in male EAE, in turn leading to changes in WDR neuron spine density. What was not investigated in this study was the specific shape of dendritic spines or relocation of spines along dendritic branches. Spine plasticity in various forms is heavily implicated in altering functional neuronal circuitry and ultimately contributing to development of chronic pain states (Alvarez and Sabatini, 2007; Tan et al., 2009, 2012). These parameters are worth considering for future studies. Furthermore, it is important to note that further research needs to be done in order to confirm results shown in exploratory sub-group analyses.

4.7 Sex Differences in Mechanism of Action

While this study does not provide a direct causal link between sex-specific plasticity, immune functions and pain in EAE, it raises interesting questions for further study. Findings from this work support evidence of sex-specific mechanisms by which tactile hypersensitivity is mediated in the spinal cord. Female mice display robust inflammatory changes at early stages of EAE, without significant changes in deep dorsal horn neuronal morphology. On the other hand, male EAE mice exhibited more prominent changes in axon injury and neurodegeneration. Furthermore, a subset analysis of male EAE mice that did not exhibit pain behaviours appear to

reflect changes in presumed WDR branching and spine density measures. In non-responding males, a decrease in average dendritic branch length and spine density relative to pain animals may serve as a protective mechanism against developing chronic pain to specific sensory modalities. This finding is consistent with the idea that aberrant spine plasticity in dorsal horn neuronal circuitry can lead to increased pain sensitivity (Labrakakis et al., 2011; Kolb and Teskey, 2012). Perhaps prominent axonal damage and plasticity changes observed in EAE males is represented as consistent, bilateral responses to tactile stimulation, while females are less affected in this regard. Moreover, it is possible that WDR and other projection neurons play a more significant role in modulating dorsal horn circuitry that ultimately lead to pain sensation in males. These findings suggest tactile hypersensitivity in male mice with EAE may rely more heavily on neurodegenerative and plasticity-related mechanisms compared to female EAE mice, in addition to the immune system dysregulation prominent in both sexes. However, the entire mechanistic action is much more complex when taking into consideration simultaneous gliosis and top-down modulation from supraspinal areas that occurs in the spinal cord. More work specifically addressing the role of WDR neurons in modulating pain phenotypes would need to be done to validate this line of reasoning.

Conclusion

This study offers insight on sex differences in inflammation, plasticity and pain within the EAE model. Though it does not provide a direct causative mechanism on how pain is differentially driven between sexes, results from this study may indicate future sex-specific targets that are worth investigating their functional role in pain circuitry. We believe that EAE-related pain hypersensitivity in males is associated with abnormal neuroplasticity changes in the spinal cord dorsal horn, a key pain processing area within the CNS. Furthermore, we discovered morphological and inflammatory differences in the spinal cord associated with development of pain between female and male mice with EAE, supporting the idea of differentially regulated pain pathways between sexes. This study was not designed to differentiate between EAE mice that do or do not demonstrate pain behaviours and therefore future investigation is encouraged with a more appropriate experimental design. The laterality of nociceptive behaviour phenomenon seen in females raises an interesting follow-up question of whether this effect persists at a chronic time point in the disease. It is important to appreciate the unending complexity of chronic pain states and to pursue future studies which further attempt to disentangle the mechanism driving sex differences in chronic pain.

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