University of Alberta

The glutamate transporters' role in neuropathic pain and cognitive deficits in an animal model of multiple sclerosis

by

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Abstract

Multiple Sclerosis (MS) is a chronic disease of the central nervous system characterized by demyelination, inflammation and axonal injury. Chronic pain and cognitive deficits affect a large percentage of MS patients. While a number of animal models are available to study the pathophysiology of MS, studies determining the profile of the above-mentioned symptoms associated with MS in these models are minimal. The purpose of this thesis was to characterize both behavioural and cellular changes in sensory and cognitive processes of the MOG_{35-55} EAE mouse model of MS.

In chapter 2, I characterized the changes in pain sensitivity that arises in a chronic relapsing model of EAE. I found that female C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG_{35-55}) develop a robust allodynia to both cold and tactile stimuli

In chapter 3, work was undertaken to determine the underlying mechanisms that generate neuropathic pain in the MOG_{35-55} EAE model. Additionally, I was interested in how MOG_{35-55} EAE mice respond to a persistent noxious stimulus. Mice with EAE showed a significant decrease in elicited pain behaviours in response to subcutaneous injection of formalin. I demonstrated that these effects are mediated by decreased glutamate transporter expression associated with the disease.

My experiments in Chapter 4 addressed changes in cognitive ability across different severities of EAE to determine if altered pain sensitivity is also associated with behavioural signs indicative of cognitive impairment in this model. I also used

the β-lactam antibiotic ceftriaxone, an agent know to increase glutamate transporter levels *in vivo* to determine if I could attenuate allodynia and NOR deficits by increasing glutamate transporter activity. Ceftriaxone prevented tactile hypersensitivity and normalized performance in the NOR assay in EAE mice

This work validates the use of the mouse MOG_{35-55} EAE model for studying sensory and cognitive changes in a laboratory setting. Furthermore, the results suggest that the glutamate transporter system may be in ideal target for treatment of these changes in patients suffering from similar sensory and cognitive deficits.

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AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA one way analysis of variance
BBB blood brain barrier
c complementary
Ca calcium
CAMs cell adhesion molecules
CCL Chemokine (C-C motif) ligand
CD cluster of differentiation
CFA complete freund's adjuvant
CGRP calcitonin gene-related peptide
CNP central neuropathic pain
CNS central Nervous System
DHK dihydrokainic acid
DNA deoxyribonucleic acid
DRG dorsal root ganglion
EAAT Excitatory amino-acid transporter
EAE experimental autoimmune encephalomyelitis
EC endothelial Cell
ERK extracellular signal-regulated kinase
FOS c-FOS protein
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GFAP glial fibrillary acidic protein
IFN interferon
IL interleukin
IP intraperitoneal
K^+ potassium
KA kainate
LPS lipopolysaccharide
m messenger
MAPK mitogen-activated protein kinase
MBP myelin basic protein
MCP moncyte chemotactic
Mg^{2+} magnesium
mGluR metabotropic glutamate receptors
MMP matrix metalloproteinases
MOG_{35-55} Myelin Oligodendrocyte Glycoprotein
MRI magnetic resonance imaging
MS Multiple Sclerosis
MS-153 [R]-[-]-5-methyl-1-nicotinoyl-2-pyrazoline
Na^{2+} sodium
NMDA N -Methyl- D -aspartic acid receptor

NOR novel object recognition
p phosphorylation
PBMC peripheral blood mononuclear cell
PCR polymerase chain reaction
PK protein kinase
PLP proteolipid protein
PNS peripheral nervous system
RM repeated measures
RNA ribonucleic acid
ROS reactive oxygen species
rpm rotations per minute
STC spinothalamic tract cells
TBOA DL-threo-beta-Benzyloxyaspartate
TMEV Theiler's Murine Encephalomyelitis Virus
TNF tumour necrosis factor
WD Wallerian degeneration

CHAPTER 1

General Introduction

1.1 Multiple Sclerosis

Jean-Martin Charcot first described Multiple Sclerosis (MS) in 1868 and presently it affects approximately 55,000 -75,000 Canadians(Compston and Coles, 2008, Girouard and Soucy, 2011). Three times as many women are affected as men, and MS typically presents in adults 20 to 45 years of age, making it one of the most common neurological disorders to affect young adults(Sospedra and Martin, 2005, Greer and McCombe, 2011). Approximately 50% of patients will require help walking within 15 years after the onset of the disease(Sospedra and Martin, 2005). The course of MS is highly variable and unpredictable across patients. The cause of MS is unknown, although it appears to involve a combination of both genetic factors (susceptibility) and non-genetic triggers (environmental factors, virus exposure, metabolism)(Sadovnick et al., 1996, Granieri and Casetta, 1997, Sotgiu et al., 2004, Ascherio et al., 2010). Although the clinical manifestation of the disease is different between patients, the majority of patients initially suffer from a relapsing-remitting disease course. The relapsing-remitting course is defined by periods of new symptoms or signs, followed by a symptom/sign reduction or remission. This first relapsing-remitting phase eventually develops into a secondary progressive phase, although 10% of MS patients develop initially with a primary progressive form(Sospedra and Martin, 2005). Presently, there is no therapy that cures MS.
MS is a disorder of the central nervous system (CNS) characterized by multifocal inflammation, demyelination, reactive gliosis and neurodegeneration(Bar-Or, 2008). The inflammatory process is characterized by accumulation of T cells initiating an

inflammatory response directed against myelin and other components of the CNS, resulting in CNS demyelination(Sospedra and Martin, 2005, Bar-Or, 2008, Racke, 2009). Areas of demyelination are described as lesions. An 'active' lesion is when there is a presence of myelin sheaths in the process of dissolution and degradation of products of myelin within the macrophages in the tissue. There are two types of active lesions, an acute plaque where the entire lesion is demyelinated simultaneously and chronic active lesions where the on going demyelization is restricted to a zone at the edge of the lesion(Compston and Coles, 2008). Exacerbations of MS are thought to be related to episodes of inflammation, while the formation of a glial scar by astrocytes or gliosis and axonal loss attributed to neurodegeneration are believed to result in the progression of disability.

1.1.1 T-cells in Multiple Sclerosis

Healthy individuals have myelin-reactive T cells, although they do not characteristically become active and migrate into the CNS, establishing an inflammatory response. Patients with MS however, do have myelin-specific T cells that are activated. The activated T cells in MS patients are more likely to be of the Th1 phenotype(Frohman et al., 2006). Th1 cells produce interferon (IFN)-gamma, interleukin (IL)-12 and tumor necrosis factor (TNF)-α cytokines and evoke cell-mediated immunity and phagocyte-dependent inflammation. In contrast, Th2 cell polarization is characterized by the production of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. This profile of cytokine production evokes strong antibody responses and eosinophil accumulation, furthermore Th2 cells inhibit several functions of phagocytic cells(Romagnani, 2000). Different subsets of T cells have been found to

play various roles in the pathogenesis of MS. Evidence for this initially came from similarities found between MS and its animal model EAE (see section 1.2) (Frohman et al., 2006, Johnson et al., 2007). EAE can be induced using Th1 CD4+ T cells(Johnson et al., 2007).

1.1.1.1 CD4+ T cells in Multiple Sclerosis

CD4+T cells are divided into distinct subsets including Th1 or Th2 cells and Th17 cells which produce IL-17A, IL-7F, IL-21 and IL-22. Regulatory T cells are another subset which are Foxp3 positive and produce IL-10(Romagnani, 2000). Further evidence linking Th1 CD4+ T cells in the pathogenesis of MS was that increased levels of IL-12 and IFN-y were found in the CNS of MS patients(Balashov et al., 1997). Although, this role was questioned when it was observed that mice lacking IL-12 and IFN-y signaling were still susceptible to EAE(Yeh et al., 2011).

Recent studies have now established an important role for Th17 cells in mediating disease induction. IL-17 producing CD4+T cells are associated with the development of disease and mice lacking factors involved in Th17 differentiation e.g. IL-6 are resistant to EAE. However, neutralization of the IL-17A receptor did not prevent EAE completely, which suggests that other molecules contribute to disease pathogenesis(Komiyama et al., 2006).

1.1.1.2 CD8 T Cells in Multiple Sclerosis

Another immune cell population that has been implicated to have a role in MS are CD8+ T cells. They are found to be prominent in the inflammatory infiltrate in MS lesions and have been reported to recognize myelin antigens in MS patients, as well as playing a role in the breakdown of the blood brain barrier (BBB)(Johnson et al.,

2007, McFarland and Martin, 2007). B cells may also play a role in MS as it has been suggested that they may secrete antibodies that recognize and play a role in myelin breakdown(Nikbin et al., 2007). It is evident that different cells of the immune system play a major role in the pathogenesis of MS, although presently no individual cell population has been observed as the key factor in development of MS.

1.1.2 The Blood Brain Barrier (BBB)

The entrance of activated immune cells from the periphery into the CNS in MS is associated with disruption of the BBB(Engelhardt, 2006). The BBB acts as an initial line of defense and it is formed by astrocytic end-feet, basal membranes and endothelial tight junctions providing a semi-permeable barrier to the CNS(Engelhardt and Ransohoff, 2005). In healthy individuals the BBB prevents the entrance of immune cells from the periphery into the CNS. In inflammatory states such as MS the BBB becomes more permissive to entering T lymphocytes. It is not clear if disruption of the BBB comes before immune cell infiltration or is a consequence of perivascular leukocyte accumulation(Engelhardt, 2006). In MS, immune cells have been shown to express inflammatory cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) which have all been associated with increasing their ability to influence the integrity of the BBB and gain access into the CNS either directly or indirectly(Larochelle et al., 2011).

1.1.3 Cytokines and chemokines in Multiple Sclerosis

Cytokines such as TNF-α and IFN-γ, are elevated in peripheral blood mononuclear cells (PBMCs) as well as in serum of MS patients and levels of TNF-α have been shown to correlate with disease activity(Navikas et al., 1996, Ozenci et al., 2000).

Endothelial cells (EC) of the BBB express receptors for both of these cytokines and increases in TNF-α levels have been shown to increase the expression of the IFN-γ_ receptor in EC-BBB cells(Kallmann et al., 2002). These two cytokines modulate the expression of different cytokines, chemokines and cell adhesion molecules (CAMs). BBB-EC's influence the expression of different chemokines such as MCP1/CCL2 and CXCL10. These chemokines promote adhesion of leukocytes to ECs and migration of leukocytes across the BBB. These chemokines play a direct role in the increase of immune cell infiltration into the CNS in MS(Chui and Dorovini-Zis, 2010). In MS they are produced by activated microglia and macrophages during myelin phagocytosis and development of demyelinating lesions(van der Goes et al., 1998).

1.1.4 Reactive Oxygen Species (ROS) in Multiple Sclerosis

In MS patients there is a shift in the balance of ROS products, such as H_2O_2, O_2- over antioxidants(Greco et al., 1999). High levels of free radicals have been shown to damage brain EC cells disrupting the junctional proteins, thus affecting BBB permeability(Van der Goes et al., 2001). Increased ROS production is also related to an increased inflammatory response and increase in TNF-α expression(Larochelle et al., 2011). The role of ROS levels has been further supported by experiments using antioxidant therapies that have resulted in a decrease of monocyte migration across BBB-ECs and suppression of clinical symptoms of EAE(Hendriks et al., 2004).

1.1.5 Matrix Metalloproteinase's (MMPs) in Multiple Sclerosis

MMPs are involved in cell migration, cytotoxicity, and inflammation. They can be secreted by activated T cells and macrophages(Parks et al., 2004). MMPs can also be secreted in response to inflammatory cytokines such as TNF-α. Levels of different

MMPs levels are increased in the serum of MS patients. In the EAE model, expression of active MMPs by T cells, monocytes and dendritic cells are required for their migration across the BBB into the CNS(Larochelle et al., 2011). MMPs have also been shown to contribute to BBB disruption by degradation of the junctional complex proteins(Rosenberg and Yang, 2007).

Once access is gained across the BBB by immune cells as a result of the above-mentioned mechanisms, CNS inflammation is promoted which favors further leukocyte infiltration. The entry of pro-inflammatory leukocytes into the CNS can trigger the events leading to neuroinflammation and BBB disruption(Larochelle et al., 2011).

As previously touched upon, cytokines and chemokines play an important role in the pathogenesis of MS, in the on-going recruitment of activated immune cells into the CNS and the disruption of the BBB. In MS there is a parallel upregulation of different pro-inflammatory cytokines including IL-6, TNF and IL-1ß (Martins et al., 2011), there is also upregulation of the chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2)(Conductier et al., 2010).

1.1.6 IL-6

One of the roles of IL-6 is to promote a proinflammatory milieu by inducing Th17 cell differentiation. IL-6 has also been found to have a role in promoting the maintenance of IL-17 secreting T lymphocytes in inflamed tissues(Kolls and Linden, 2004). In acute and chronic active plaques from the brains of MS patients IL-6 is present. The IL-6 found in these samples was largely associated with resident cells in the CNS at these sites of ongoing demyelination and immune activation

suggesting a major role for IL-6 in the pathogenesis of MS(Maimone et al., 1997). In EAE models where IL-6 was blocked, the development of EAE was suppressed(Samoilova et al., 1998). It is postulated that by blocking IL-6 and preventing the development of Th17 and Th1 cells there is a decreased infiltration of T cells into the CNS, explaining the suppression of EAE(Samoilova et al., 1998).

1.1.7 TNF-α

TNF-α has been observed to exert multiple effects including roles in immunity, inflammation, and control of cell proliferation, differentiation and apoptosis(SY Lim, 2010). Autopsies of MS patients have found high levels of TNF-α at active MS lesion sites(Selmaj et al., 1991). In the CSF of MS patients increased levels of TNF-α were found to correlate with disease severity and rate of neurological deterioration(Sharief and Hentges, 1991). In MS patients, there are normal serum levels of TNF-α, suggesting that it, like IL-6 is produced by cells intrinsic to the CNS(Selmaj et al., 1991). In animals treated with TNF antibodies, the development of EAE was prevented and the protective effect was observed for several months after the initial suppression treatment(Ruddle et al., 1990). In mice deficient of TNF-α, the onset of clinical disease was found to be significantly delayed, however after disease onset, the progression and severity of the disease was not different from the wild type EAE mice(Probert et al., 1995). This suggests a role for TNF-α in the initiation of EAE as opposed to involvement in disease progression or from recovery of the acute clinical disease. However, treatments in MS patients that involved neutralizing TNF-α have not been successful, and resulted in an increase in the disease severity(Group, 1999). It has been proposed that more selective agents,

perhaps specific to different TNF-α receptors are required to see the benefits of targeting TNF-α as a treatment strategy in MS.

1.1.8 IL-1ß

IL-1ß is a pro-inflammatory cytokine that like IL-6 and TNF-α exerts pleiotropic effects. In MS, IL-1ß is expressed by microglial cells and infiltrating monocyte/macrophages in acute lesions and in the white matter(Carpintero and Burger, 2011) Rats treated with an IL-1ß receptor blocker during induction of EAE, developed milder disease severity profiles(Burger et al., 2009). Mice that are IL-1 α /ß deficient have great resistance to EAE and also have a significant reduction in the clinical disease severity if EAE is induced(Dinarello, 2011). This evidence suggests an important role for IL-1ß in MS pathogenesis.

1.1.9 MCP-1/CCL2

Elevated expression of MCP-1/CCL2 has been found in acute and chronic MS plaques(Conductier et al., 2010). In several EAE models it has been found that the animals over express MCP-1/CCL2, with levels increasing just prior to symptom onset and peaking at the height of clinical disease stages(Proudfoot et al., 2008). Furthermore, mice that are lacking the MCP-1/CCL2 receptor, CCR2 do not develop clinical signs of EAE. These mice had an absence of CNS inflammation and other chemokine and proinflammatory cytokine expression(Huang et al., 2001). This suggests that the MCP-1/CCR2 receptor interaction is needed to produce and active lesion, which is characterized by production of pro-inflammatory chemokines that in turn amplify the local inflammatory response.

A large battery of cytokines and chemokines result in the immune-mediated inflammation that is witnessed in MS. Although many different cytokines and chemokines including the above mentioned have been described in lesions in MS and have been found to be increased in different animal models of MS, the exact pattern of cytokine expression in MS is still elusive. See figure 1.1 for a representation of the immune response in MS.

1.1.10 Axon Pathology

Along with inflammatory demyelination in MS, degeneration of demyelinated CNS axons is now considered a major hallmark of the disease. Axonal degeneration has been established as an important component for disease progression(Compston and Coles, 2008, Stys, 2010). Loss of axonal integrity in MS brains has been correlated with disease severity(Tourbah et al., 1999). Axonal loss in MS is not restricted to areas of inflammation. It is unclear if axonal damage is secondary to demyelination or if axons are degenerated by the same degenerative mechanism that is affecting the myelin(Stys, 2010). Axonal damage has also been suggested to result from a secondary phenomenon, such as a result of increased excitotoxicity from increased glutamate levels observed in MS(Pampliega et al., 2011).

Recently it has been proposed that the neurodegeneration associated with MS may be the initiating factor in the disease, as opposed to the long held belief that the degeneration observed in MS is a result of the initial neuroinflammation component(Stys, 2010). Although it is not clear which, if any of these mechanisms initiate the disease, it is accepted that both neuroinflammation and neurodegeneration contribute greatly to the pathogenesis of MS.

1.2 Animal Models of MS

1.2.1 Experimental Autoimmune Encephalomyelitis

The EAE animal model of MS has been in use for over 50 years. It can be induced in several different species either by direct immunization which is referred to as active transfer or passive transfer of T cells specific for selective autoantigens(Hart et al., 2011). The most commonly used antigens are made from small fragments of myelin peptides such as myelin basic protein 84-104 (MBP84-104); proteolipid protein 139-151 (PLP139-151), or myelin oligodendrocyte glycoprotein 35-55 (MOG35-55)(Mix et al., 2008, Miller et al., 2010, Hart et al., 2011). In addition to induction using autoantigens, pertussis toxin is also often injected to create holes in the BBB and result in an increased inflammatory response(Mix et al., 2008). The disease onset in EAE occurs from 5-14 days post injection, and follows an initial disease profile similar to MS(Mix et al., 2008). EAE models share many of the same pathological features as MS, including widespread CNS inflammation, demyelination and locomotor impairments(Hart et al., 2011). The severity of these pathologies can vary depending on the type of autoantigen used, how EAE is induced, or the genetic background of the animal used(Lassmann, 2010). EAE can be induced to mimic both a relapsing-remitting MS and chronic MS phenotype(Olechowski et al., 2009). EAE animals that are used to generate a relapsing-remitting disease course show intermittent periods of improved clinical symptoms throughout the disease course. Whereas animals induced with a chronic EAE phenotype, do not have these periods

of symptom recovery and progress in severity over the disease course(Olechowski et al., 2009).

1.2.2 Theiler's Murine encephalomyelitis virus (TMEV) model

Another common animal model used to study multiple sclerosis is the TMEV model, where susceptible rodent strains develop a demyelinating disease as a result of intracerebral infection with TMEV that is chronic, and consistent with MS is characterized histologically with immune cell infiltrates into the CNS(Tsunoda and Fujinami, 2010). Unlike EAE the TMEV model always mimics a chronic-progressive disease course that lasts the entire lifespan of susceptible mice. Because of this, it has been used to study the role of axonal injury, disability independent of demyelination, and the significance of remyelination(Fuller et al., 2004). TMEV has also been found to mimic several MRI findings of the human disease(Denic et al., 2011).

1.2.3 Toxin Induced Demyelination Models

The exposure to different oligodendrocyte toxins including cuprizone or direct injection of ethidium bromide or lysolecithin into the CNS has been shown to produce demyelination(Rodriguez, 2007, Acs and Kalman, 2012). These models are utilized to study the mechanisms of focal demyelination and remyelination, as they do not attempt to mimic MS as a disease.

As of yet there is no single animal model that mimics human MS, although the available animal models do represent selective aspects of the human disease and can be used to compliment each other. The appropriate use of the animal models of MS depends on the specific research question one has.

1.3 Cognitive Deficits and Multiple Sclerosis

Impairments in cognitive functioning are now recognized as being highly prevalent in the MS population although they have not been well studied until recently. Specifically, MS patients seem to have impairment with memory, speed processing, executive function, attention and concentration domains(Guimaraes and Sa, 2012). Thirty years ago, estimates of the prevalence of cognitive impairments in the MS population was reported as 3 %. Currently, estimate of prevalence of these symptoms range from 43-72%(Ferreira, 2010). Cognitive deficits can appear early and late in the disease course, although they tend to increase in severity as disease severity increases(Huijbregts et al., 2004). It has been established by different groups that impairments in processing speed and memory are often present in very early stages of MS(Schulz et al., 2006, Simioni et al., 2007, Guimaraes and Sa, 2012). The presence of cognitive deficits has been associated with a decreased quality of life index(Cutajar et al., 2000, Baumstarck-Barrau et al., 2011). Interestingly, in MS patients with poorer performances on cognitive tasks there is a correlation with an increase in IL-6 production by PBMCs, suggesting that this cytokine may play a potential role in the cognitive impairments seen in MS(Patanella et al., 2010). Despite the identification of different cognitive impairments in MS, and the implicated roles of different cytokines the underlying mechanisms are not well studied or understood.

1.4 Pain and Multiple Sclerosis

In clinical populations there is a strong association between chronic pain and cognitive dysfunction. Pain is now accepted as a common symptom among MS patients, affecting about one in five patients at disease onset, and as many as half of patients at any given point in their disease(Svendsen et al., 2003, Svendsen et al., 2005). Similar to cognitive impairments, pain in MS patients is associated with a significantly decreased health related quality of life index compared to the general population; the major impairments include decreased physical and emotional functioning(Khan and Pallant, 2007). Several different types of pain are associated with MS including continuous central neuropathic pain, intermittent central neuropathic pain, musculoskeletal pain, and mixed neuropathic and non-neuropathic pain(O'Connor et al., 2008). Men and women who have MS do not appear to differ in their susceptibility to pain symptoms, although it has been suggested that woman have a greater severity of pain(Osterberg et al., 2005). It has also been suggested that pain symptoms increase as the disease severity increases(O'Connor et al., 2008).

1.4.1 Central Neuropathic pain in MS

Central neuropathic pain, which results from central nervous system lesions, is thought to affect about 30% of patients suffering from pain in MS(Osterberg et al., 2005). Central neuropathic pain is defined as pain in a neurologic distribution with altered sensation and no history or clinical evidence of peripheral neuropathy(Osterberg et al., 2005). In one study, one-third of patients with central

neuropathic pain have two or more central neuropathic pain types and locations. In most patients with neuropathic pain, pain affected the lower extremities(Nick et al., 2012). Patients with central neuropathic pain commonly have abnormal sensory exams; including lowered pain pressure thresholds when compared to control patients who did not suffer from MS related pain(Osterberg et al., 2005, Svendsen et al., 2005). Other studies have found that MS patients also present with symptoms of both cold and mechanical allodynia(Osterberg et al., 2005). Although central neuropathic pain affects such a substantial amount of MS patients its pathophysiology is not well understood and because of this there are no specific treatments. Given the high prevalence of pain in MS patients additional studies are needed to determine not only the natural history, but also additionally the mechanisms that underlie pain in MS.

1.5 Neuropathic Pain general mechanisms

Neuropathic pain is thought to arise due to changes in neural processes in the CNS(Woolf and Salter, 2000). These changes are observed experimentally as increases in the activity of neurons involved in pain transmission in the spinal cord. The changes in the excitability and activity of spinal neurons is referred to as central sensitization(Woolf and Thompson, 1991). Central sensitization is believed to be a primary mechanism of neuropathic pain in MS patients(Svendsen et al., 2005). Central sensitization is critically dependent on alterations in glutaminergic signaling(Coderre, 1992, Chizh and Headley, 2005, Bleakman et al., 2006). The

activation of glutamate receptors has been strongly implicated in the generation of central sensitization(Coderre, 1993). These include the iontropic glutamate receptors (iGluRs); N-methyl-D-aspartate (NMDA), 1-amino-3-hydroxyl-5-methyl-4-isoaxolepropionic acid (AMPA) and kainate (KA)(Dickenson and Sullivan, 1987, Yoshimura and Jessell, 1990, Woolf and Thompson, 1991).

1.5.1 NMDA receptor

The NMDA receptor has been a focus of neuropathic pain research for decades due to its physiological role in pain processing(Woolf and Thompson, 1991). In mammalian cells it is composed of two different subunits NR1 and NR2. Two of each subunit make up its hetero tetramer structure(Dingledine et al., 1999). The NMDA receptor acts as a non-specific cation channel, allowing the passage of Ca^{2+} and Na+ into the cell and K+ out. In order for the NMDA receptor to be activated, it has to be bound to glutamate and the Mg^{2+} block has to be removed with depolarization of the cell. Because of the gating properties of the Mg^{2+} block, most NMDA receptors are not active under normal conditions(Paoletti and Neyton, 2007). However, sustained noxious stimuli that are associated with tissue damage in neuropathic pain and subsequent postsynaptic depolarization can disrupt this Mg^{2+} block and activate the NMDA receptors. This allows an influx of Ca^{2+} which could lead to the phosphorlyation of the NMDA receptor, prolonging the excitability of spinal cord neurons(Woolf and Thompson, 1991). Spinal cord administration of an NMDA receptor antagonist has been shown attenuate hyperalgesia and allodynic pain behaviours(Ma et al., 1998). It has also been shown at a cellular level, that an antagonist to the NMDA receptor can prevent central sensitization of spinothalamic

tract cells (STC)(Randic et al., 1990, Dougherty and Willis, 1991).

Central sensitization of STCs also has found to be dependent on the activation of several different protein kinases, including PKC and PKA. PKC and PKA *in vitro* have both been shown to produce long-lasting increases in excitatory responses in dorsal horn neurons(Chen and Huang, 1991, Lin et al., 1996, Sluka et al., 1997). In slice preparations of neurons after PKC injection, it has been found that responses to NMDA are enhanced(Chen and Huang, 1991). This change in NMDA receptor function and the consequent response can be explained by an increased in the probability of channel openings and a decrease in the Mg^{2+} block of the NMDA receptor channels(Hatt, 1999). One mechanism that can be attributed to this change in NMDA receptor function is the phosphorylation of the NMDA receptor. PKC has been shown to phosphorylate Ser-890, 896 specific sites of the NR1 subunit and PKA at the Ser-897 site(Tingley et al., 1997). Increases in phosphorylation of the NR1 subunit have been shown to contribute directly to the development of central sensitization of STCs.

1.5.2 Metabrotropic G-protein coupled glutamate receptors

A family of metabrotropic G-protein coupled glutamate receptors (mGluRs) are also implicated in this process. Receptor antagonists for both types of receptors have been utilized experimentally to determine their efficacy for treating neuropathic pain(Young et al., 1995). Antagonism of ionotropic GluRs and mGluRs can alter neuropathic pain behaviours in animal models of peripheral nerve or spinal cord injury(Bennett et al., 2000, Mills et al., 2002, Gwak et al., 2007). However, the utilization of these agents in humans is limited, since antagonism of these receptors

is associated with a number of unwanted side effects such as, ataxia, hallucinations and sedation(Fundytus, 2001, Brown and Krupp, 2006).

1.5.3 Glial cells and neuropathic pain

It has now been established that glial cells, specifically astrocytes and microglia play an important role in the development and maintenance of neuropathic pain(Milligan and Watkins, 2009). Astrocytes and microglia are able to modulate neuronal synaptic function and excitability, allowing them to play various roles in pain facilitation(Halassa et al., 2007, Pocock and Kettenmann, 2007). Glial activation in the spinal cord underlies many different pain syndromes from various causes including, peripheral nerve inflammation, diabetic neuropathy and spinal cord trauma and inflammation(Jha et al., 2012). Activated glia cells release pro-inflammatory cytokines such as IL-1ß, IL-6 and TNF-α that can lead to neuropathic pain states. IL-1ß and TNF-α released from activated astrocytes increase the conductivity and amount of NMDA receptors on the surface of spinal cord neurons, which in turn increases neuronal excitability(Milligan and Watkins, 2009). IL-ß, TNF-α, and IL-6 have also been shown to play a direct role in mediating spinal cord dorsal horn central sensitization(Kress, 2010). Drugs that inhibit both astrocyte and microglial function, have been shown to attenuate the induction and maintenance of allodynia and hyperalgesia in pathological pain models(Wieseler-Frank et al., 2004). These findings suggest that early microglial activation and the persistent activation of astrocytes following microglial activation contribute both to the induction and maintenance of neuropathic pain.

When microglia are activated they undergo a morphological change from a ramified shape to an amoeboid shape and undergo an increase in proliferation. Using microglia activation markers such as OX-42 it has been established that microglia activation precedes astrocyte activation in pathological pain conditions such as nerve injury and peripheral inflammation(Schomberg and Olson, 2012). As the initial microglia activation starts to decrease, astrocytic activation is present and neuropathic pain behaviours are still observed(Milligan and Watkins, 2009). In a spinal nerve ligation model of neuropathic pain, this temporal pattern was confirmed. Activation of both mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK) signaling proteins occurred in microglia first, with activation of these factors in astrocytes after(Colburn et al., 1999). Inhibition of microglia activation with the drug minocycline, a tetracycline antibiotic has been shown to prevent the induction of pathological pain. When minocycline is administered when hyperalgesia and allodynia is present following nerve injury or inflammation, it does not typically reverse these symptoms(Raghavendra et al., 2003).

1.5.4 Cytokines and Chemokines in Neuropathic pain

The release of proinflammatory cytokines from activated glia cells as previously mentioned play an important role in the induction and continued maintenance of neuropathic pain conditions. Blocking proinflammatory cytokines or administration of anti-inflammatory cytokines in different neuropathic pain animal models has been shown to reduce neuropathic hyperalgesia(Sommer and Kress, 2004).

1.5.4.1 IL-1ß

IL-1ß is produced and secreted in many different pathological conditions that are associated with neuropathic pain(Watkins and Maier, 1999). Direct injection of IL-1ß into the spinal cord of rats, has been shown to lower mechanical nociceptive thresholds and results in hyperalgesia. Antagonism of the IL-1 receptor has been shown to reduce pain behavaiours in mice with experimental neuropathy(Cunha et al., 2000). IL-1ß sensitization can be reduced by antagonizing PKC, suggesting that IL-1ß may play an indirect role in the increased phosphorylation of dorsal horn neurons through increased activation of the NMDA receptor(Sommer and Kress, 2004).

1.5.4.2 IL-6

Similar to IL-1ß, IL-6 is upregulated in different pathological states associated with neuropathic pain. Significant increases in IL-6 serum levels have been detected in patients with neuropathies, burn injury, autoimmune and chronic inflammatory conditions(Sommer, 2001). Hypersensitivity to touch is common and similar in all these conditions at the site of affected tissue(Sommer and Kress, 2004). Injection of IL-6, either intraplantar or intrathecally induces hyperalgesia and allodynia in rats(De Jongh et al., 2003). In IL-6 knockout mice there is a reduction in nerve injury related hyperalgesia(Ramer et al., 1998, Murphy et al., 1999). Also neutralization of IL-6 has been shown to inhibit lipopolysaccharide (LPS) induced hyperalgesia(De Jongh et al., 2003).

1.5.4.3 TNF- α

TNF-α, plays a major role in the initiating cascade of activation of other cytokines, including IL-1ß and IL-6 and growth factors in the inflammatory response. Different

groups have correlated increased tissue levels of TNF-α and pain in a number of diseases associated with neuropathic pain(Sommer and Kress, 2004). Similar to IL-1ß and IL-6, direct injection of TNF-α into rats has been shown to induce mechanical allodynia and thermal hyperalgesia(Schafers et al., 2003). In the rat sural nerve specifically, it was shown that injected TNF-α lowers the mechanical activation thresholds of C nociceptors. Blocking TNF-α reduces hyperalgesia in both rat and mouse models of painful neuropathy(Sommer and Kress, 2004). Injured neurons have also been shown to be more susceptible to the effects of TNF-α. In rat DRG that was previously ligated, less TNF-α was needed to induce allodynia, and this allodynia was induced significantly faster then with an uninjured DRG(Schafers et al., 2003).

1.5.4.4 MCP-1/CCL2

Similar to the role of pro-inflammatory cytokines in neuropathic pain conditions, the chemokine MCP-1/CCL2 is upregulated in different neuropathic pain conditions and has also been shown to play an important role(Thacker et al., 2009). Administration of MCP-1/CCL2 directly into the spinal cord results in the development of pain behaviours. MCP-1/CCL2 has also been shown to influence sensory neurons directly and play an important role in pain regulation. MCP-1/CCL2 and its receptor are both increased in DRG neurons following nerve injury(Sun et al., 2006). This increase in MCP-1/CCL2 has been associated with an increase in the activation of spinal microglial cells, suggesting its role in the initiation of the neuropathic pain process(Hingtgen, 2006, Thacker et al., 2009). Activation

astrocytes have also been shown to release MCP-1/CCL2, maintaining the neuropathic pain environment(DeLeo et al., 2004).

Proinflammatory cytokines and chemokines have been shown to contribute to neuropathic pain states, although the exact relationship between the chemokine-cytokine network and neuropathic pain such as the regulation of these processes still needs to be established.

1.5.5 Wallarian degeneration

Many neuropathic states are associated with Wallarian degeneration (WD) and demyelination in the periphery(Ramer et al., 1997). WD occurs when the nerve fibre's connectivity is disrupted. This can occur as a result of traumatic, toxic, ischemic or metabolic events. Axonal injury stimulates a series of events that include, breakdown of the BBB and blood nerve barrier, proliferation of Schwann cells, macrophage activation, and elevations of cytokine production(Ramer et al., 1997, Gaudet et al., 2011). These changes are meant to stimulate new regrowth of the axon and support axonal regeneration, these changes are also involved in the induction and maintenance of neuropathic pain states at the same time(Dubovy, 2011).

Following nerve damage, the process of WD begins with degradation of the axoplasm and axolemma, as well as the myelin sheath distal to the site of nerve lesion. As a result of this degradation, macrophages are recruited to clean up the debris. The clearance of the myelin sheath is important, because of the presence of axon growth inhibitors in peripheral nerve myelin(Perry and Brown, 1992). Schwann cells play a role in recruiting macrophages to clean up myelin debris by

releasing inflammatory mediators to recruit more macrophages to the injury site (Shamash et al., 2002). These same inflammatory mediators may also work to sensitize rescued or regenerated neurons. This inflammatory reaction during WD could explain the neuropathic pain induction that happens simultaneously. It is also established that injured afferent axons produce abnormal spontaneous discharges that can also favour neuropathic pain conditions. Nerve injury also has been shown to cause molecular changes in the dorsal root ganglion (DRG), which can result in a change in expression of different cytokine or chemokine concentrations(Dubovy, 2011). WD may therefore be responsible for signaling alterations of different inflammatory markers in the DRG. Although most of this work on WD and demyelination and their contribution to neuropathic pain states has been done in the PNS, it is still a question as to whether the same mechanism of neuropathic pain generation is conserved in the CNS. This may be the case in disorders where demyelination and axonal degeneration are significant pathological features such as MS.

1.6 Glutamate Transporters

The activation of glutamate receptors is dependent on the amount of extracellular glutamate available at the synapse(Danbolt, 2001). The levels of extracellular glutamate that can be utilized are regulated by a class of Na+-dependent glutamate transporters that have been found to be expressed in both neurons and glial cells. To date, five excitatory amino acid transporters (EAATs) have been cloned as with

their human homologues; EAAT-1 (also known as GLAST), EAAT-2 (GLT-1), EAAT-3 (EAAC-1), EAAT-4 and EAAT-5. EAAT-1 and EAAT-2 are predominantly localized in glial cells, whereas EAAT-3 is primarily expressed in neurons(Danbolt, 2001).

1.6.1 Biophysical properties of Glutamate Transporters

The glutamate transporters crystal structure appears as homomers that are made up of 3 different subunits(Jiang and Amara, 2011). These transporters use free energy stored in ion/solute gradients to drive substrate movement. The transport cycle includes co-transport of one glutamate with 3 sodium ions and 1 proton into the cytoplasm and counter transport of 1 potassium ion(Danbolt, 2001, Maragakis et al., 2004, Tzingounis and Wadiche, 2007). This process generates a net influx of two positive charges for each transport cycle, creating a large gradient of glutamate across the cell membrane. This transporter cycle allows for an outward and inward conformation of the transporter. The outward conformation is when the glutamate-binding site is exposed to the extracellular environment, the inward conformation is when this site is exposed to the cytoplasm. Binding of potassium to the inward conformation triggers a change in conformation back to the outside. This change of conformation is what allows for the accumulation of glutamate against a concentration gradient(Tzingounis and Wadiche, 2007).

Manipulation of the glutamate transporter system has contributed greatly to our understanding of the role that the transporters play in the CNS. Inhibiting the glutamate transporter system results in an increase of glutamate-mediated injury as a result of the increase of glutamate concentration at the synapse. Different analogs of DL-threo-Bbenzyloxyaspartate (TBOA) have be used as transporter blockers.

Using rat hippocampal slices it was found that blocking transporter function resulted in an increase time for the removal of glutamate at the synapse(Tzingounis and Wadiche, 2007). Prolonged application of TBOA to the slices resulted in spontaneous epileptic discharges that confirmed the critical role of glutamate uptake in maintaining glutamate homeostasis(Campbell and Hablitz, 2004). A problem that is encountered using TBOA analogues is the inability for them to be specific to certain transporter subtypes(Shimamoto et al., 1998).

Conversely, increasing the expression or activity of glutamate transporters has been shown to be successful in removing glutamate from the synapse, especially when there has been an insult to the CNS and normal glutaminergic clearance has been compromised. Drugs that promote glutamate transporter activity work either to increase the catalytic activity of the transporter or to increase levels of transporter protein expression. ß-lactam antibiotics are able to increase the expression of the EAAT-2 glutamate transporter by as much as a 6 fold increase(Rothstein et al., 2005). Of the different ß-lactam antibiotics that have been tested, ceftriaxone was found to be the most beneficial in increasing EAAT-2 protein levels and was found *in vitro* to have neuroprotective properties(Rothstein et al., 2005). Compared to drugs that target other components of glutaminergic transmission such as drugs that target the different glutamate receptors, drugs targeting specific glutamate transporters are lacking. Because of this, the role of glutamate transporters under normal and pathological conditions is still not fully understood.

1.6.2 Glutamate transporters and pain signaling

In the spinal cord, EAAT-1, EAAT-2 and EAAT-3 are concentrated in the superficial layers of the dorsal horn, which is the site in which pain processing is initiated in the CNS(Vera-Portocarrero et al., 2002, Sung et al., 2003). This pattern of localization implies that transporters may play a role in the regulation of pain processing. Changes in the expression and activity of glutamate transporters in the spinal cord has significant impact on sensory transmission in the dorsal horn of the spinal cord(Liaw et al., 2005, Tao et al., 2005). Intrathecal administration of the transporter blockers (TBOA) or dihydrokainate (DHK) to normal rats leads to spontaneous pain behaviours and an increased sensitivity to both thermal and mechanical stimuli(Liaw et al., 2005).

Glutamate transporters have also been implicated in neuropathic pain models. A decrease in transporter expression was found in the dorsal horn of the spinal cord in a model of peripheral nerve injury. Peripheral nerve injury was also found to significantly reduce the uptake of spinal glutamate post operatively. A study using spinal nerve ligation found that this decrease in glutamate uptake was still present in the superficial dorsal horn as late as 6 weeks after the initial injury(Sung et al., 2003).

A physiological consequence of decreased glutamate transporter expression would be an elevation in ambient glutamate levels at the synapse. Loss of these transporters could also lead to “spillover” of glutamate into nearby or adjacent synapses and the activation of distant cells. This would result in greater excitotoxicity and increases in the activation of glutamate receptors, a primary mechanism of central sensitization.

1.6.3 "Reverse transport" and pain sensitivity

With certain experimental conditions, the down regulation of glutamate transporter expression has the opposite effects as what is described above. The transporter inhibitor trans-pyrrolidine-2,4-dicarboxylic acid or the selective knock down of EAAT-1 or EAAT-2 with antisense oligonucleotides were found to decrease pain behaviours that were evoked using zymosan induced inflammation or in response to intraplantar formalin administration(Niederberger et al., 2003, Niederberger et al., 2006). The mechanisms behind the analgesic effects in response to transporter blockade remain unknown. It has been postulated that the diminished pain behaviours in the above conditions may be through a blockade of "reverse transport" by the glutamate transporters themselves. The mechanism by which glutamate transporters exert their actions is dependent on membrane potential and specific transmembrane ion gradients. With pathological conditions, metabolic insults can result in a disruption of intracellular energy stores and perturbation of ionic gradients in a way that the transporters, instead of removing extracellular glutamate, begin to "reverse transport" and actually release glutamate into the extracellular space(Rossi et al., 2000). This phenomenon has been documented in cases of brain ischemia and in the acute phase after spinal cord injury. (Rossi et al., 2000, Vera-Portocarrero et al., 2002)

1.6.4 Glutamate transporters and Multiple Sclerosis

Decreased expression of glutamate transporters has been observed in post mortem samples from patients with MS and with rats and mice with EAE(Ohgoh et al., 2002, Vercellino et al., 2007, Pampliega et al., 2008, Olechowski et al., 2009). A decrease in

glutamate transporter expression, and therefore an increase is overall glutamate in MS is consistent with the finding of that levels of glutamate are increased in the CSF of patients with MS. This decrease in glutamate transporter expression is thought to be a result in part, by the initial accumulation of proinflammatory cytokines in the first stages of the disease onset. The inflammatory insults that are considered hallmarks of MS and EAE have been shown to be associated with mitochondrial dysfunction, energy failure and altered ion exchange mechanisms (Friese et al., 2007). These metabolic insults may trigger a similar reverse transporter function in cases of MS and EAE that would lead to an overall increase in the levels of extracellular glutamate, therefore resulting in increased receptor activation, sensitization and consequently, abnormal pain sensitivity.

1.7 Purpose

The purpose of this thesis was to characterize changes in pain and cognitive behaviours in the MOG_{35-55} EAE mouse model of MS. To determine potential mechanisms behind these changes and to normalize these changes with pharmacological treatment.

Hypothesis 1: MOG_{35-55} EAE mice display neuropathic pain behaviours at different points of the disease course.

Hypothesis 2: Glutamate transporters play a role in the dsyregulation of pain behaviours in MOG_{35-55} EAE mice.

Hypothesis 3: MOG_{35-55} EAE mice display cognitive deficits early on in the disease course.

Hypothesis 4: The severity of the EAE disease course does not affect the appearance and degree of neuropathic pain behaviours and cognitive deficits at disease onset.

Hypothesis 5: Increasing the activity of glutamate transporters in MOG_{35-55} EAE mice will normalize the changes in pain behaviours and recover the loss of cognitive function in MOG_{35-55} EAE mice.

1.8 Figures

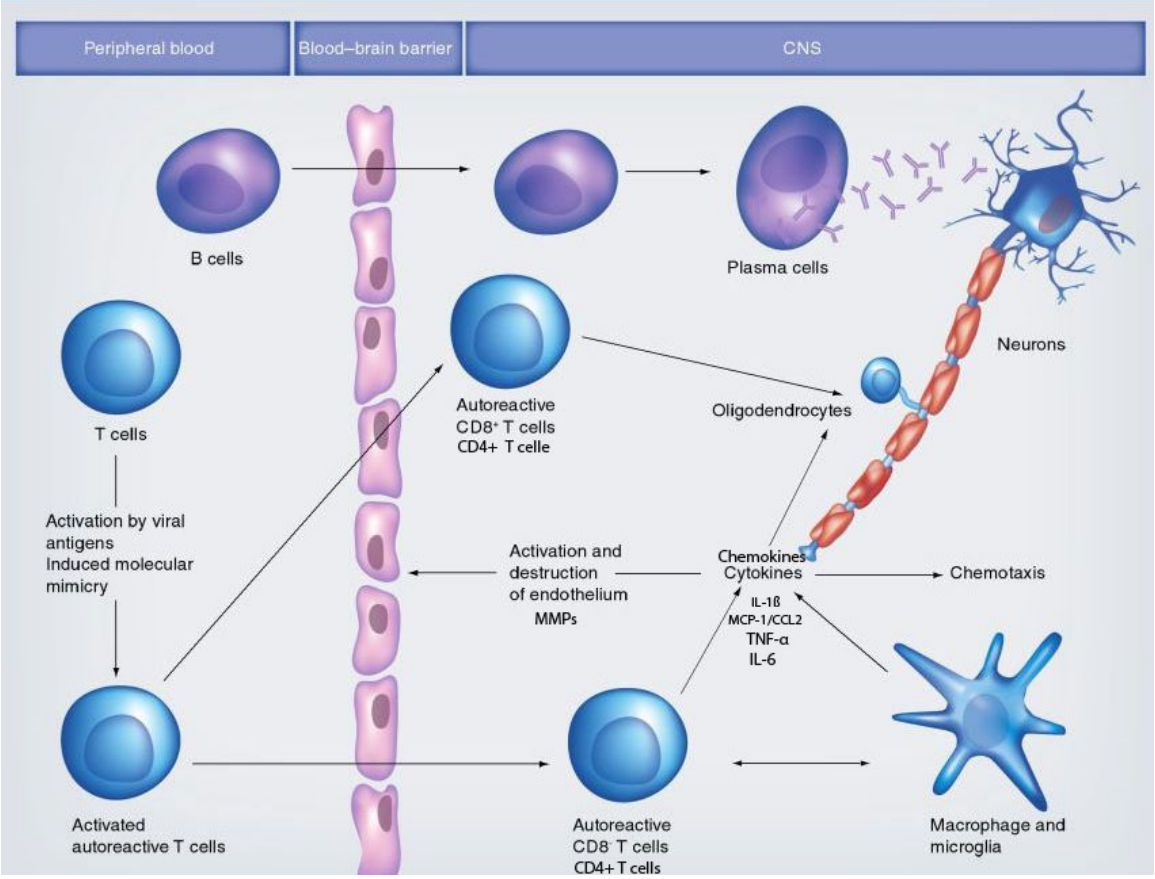

Figure 1.1
Representation of the immune response in Multiple Sclerosis, modified version of adapted figure from Delbue et al 2012.

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CHAPTER 2

Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE)

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2.0 Introduction

Chronic pain is a common, but little understood symptom associated with the autoimmune disease Multiple Sclerosis (MS) (Clifford and Trotter, 1984, Moulin et al., 1988, Archibald et al., 1994, Svendsen et al., 2003). It has been estimated that approximately 50-80% of MS sufferers experience clinically significant pain at some point in the course of their disease (Osterberg et al., 2005). Neuropathic pain, which arises due to lesions or dysfunction in the central nervous system (CNS), is the most prevalent and difficult to treat pain syndrome seen in MS patients (Svendsen et al., 2005). However, studies focused on identifying the underlying mechanisms of neuropathic pain associated with MS are lacking.

Experimental autoimmune encephalomyelitis (EAE) is an accepted animal model of MS that shares many features of the pathology seen in MS patients, including widespread CNS inflammation, demyelination and locomotor impairments (Owens and Sriram, 1995, Steinman and Zamvil, 2006, Baxter, 2007). EAE can be induced in genetically susceptible mouse strains by immunization with small fragments of myelin antigens such as myelin basic protein 84-104 (MBP_{84-104}); proteolipid protein 139-151 ($PLP_{139-151}$); or myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) (Bradl and Hohlfeld, 2003). Depending on the strain of mouse and myelin antigen used, different disease courses can be modeled that reflect the differences in symptom progression often observed in human MS. While EAE has been used effectively to study the classical neurological deficits associated with MS such as locomotor dysfunction and paralysis, there is limited data on the specific changes in somatosensory processing that occur in the model.

A recent study has described altered pain behaviours in a relapsing-remitting form of EAE in the SJL/J strain of mice after immunization with $PLP_{131-151}$ (Aicher et al., 2004). These mice show signs of thermal hyperalgesia (an increased sensitivity to noxious heat) that develops at the peak of neurological deficits and persists throughout the course of the disease (Aicher et al., 2004). While these findings are an important validation of using the EAE model to study MS-related pain, only one sensory parameter (i.e., hyperalgesia) has been published thus far. Cold and tactile allodynia (painful responses to non-painful stimuli) are also often reported from MS patients with neuropathic pain (Osterberg et al., 2005, Svendsen et al., 2005).

To address this, we have used a chronic-relapsing model of EAE in female C57BL/6 mice immunized with MOG_{35-55} and assessed changes in sensitivity to noxious heat, innocuous cold and mechanical stimuli. In addition, because inflammatory processes and reactive gliosis in the spinal cord are known to contribute to neuropathic pain behaviours in peripheral nerve and spinal cord injury models (Colburn et al., 1999, DeLeo and Yezierski, 2001, Tsuda et al., 2003, Hains and Waxman, 2006) we have also examined these responses in the superficial dorsal horn in mice with MOG_{35-55} EAE.

2.1 Materials and Methods

2.1.1 Induction of EAE. A total of 39, 10-12 weeks old female C57BL/6 mice were used. Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. All animal procedures and experiments were approved by the University of Alberta Health Sciences Laboratory Animal Services. The course and severity of symptoms in EAE are often variable between different laboratories even when using the same antigen, doses and induction protocols. Although a number of factors can account for these variations, differences in the source of where MOG_{35-55} is obtained from can be a major factor. Therefore, we induced EAE in mice with myelin oligodendrocyte glycoprotein 35-55 (MEVGWYRSPFSRVVHLYRNGK; MOG_{35-55}) obtained from two different sources to determine whether this would generate differences in disease course and symptom severity and if so, if this has any effect on pain behaviours. MOG_{35-55} peptide was obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB) (a generous gift from Dr. Chris Power, University of Alberta) and also from AnaSpec Inc (San Jose, CA). EAE was induced by subcutaneous immunization with 50 µg of MOG_{35-55} from either source (grouped as EAE "A" n=10; or EAE "B" n=10 respectively) emulsified in complete Freund's adjuvant (CFA) (1 mg/ml to a final concentration of 0.5mg/ml CFA, Sigma-Aldrich, Oakville, ON). An intraperitoneal injection of 300ng Pertussis toxin (Sigma-Aldrich, Oakville, ON) was administered at the time of induction and again 48 hours later. Control mice were treated with CFA (0.5mg/ml) and Pertussis toxin alone (n=10).

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

2.1.2 Behavioural testing

All behavioural testing was carried out by an experimenter blinded to the specific treatment groups. Mice were given 15-20 minutes to acclimatize to the testing apparatus for a given assay before the testing began.

2.2.1 Rotorod test: As a test of gross locomotor ability and coordination mice were placed on a rotorod at a fixed rotational speed of 16rpm. Mice were placed on the rotorod and the latency to fall off was determined. The maximum time for a trial was set at 180s. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. After disease induction mice were tested on alternating days. Each mouse had three trials and the mean latency to fall per trial was calculated.

Thermal Hyperalgesia: After disease induction mice were tested on alternate days using the Hargreaves test (Hargreaves et al., 1988). This test measures the latency to withdrawal from a noxious heat source applied to the plantar surface of the hind paws. To prevent tissue damage a cut off score of 20 seconds of applied heat was used. Withdrawal latencies were recorded three separate times for each paw and the average withdrawal latency was calculated.

Cold Allodynia: A single drop of acetone was applied to the dorsal surface of each hind paw in turn and the duration of response (lifting, guarding, licking or biting) was recorded. On a test day, each paw was tested once separated by at least 5 minutes between tests. The average duration of response from the two hindpaws was then calculated.

Mechanical Allodynia: A set of calibrated von Frey hair monofilaments were used to assess the sensitivity to punctate mechanical stimuli. Mice were placed in clear pexiglass chambers on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hind paw in ascending order of bending force (range: 0.04g-2.0g). Each hair was applied 5 times per paw and the number of nocifensive responses (vigourous shaking, prolonged lifting, licking or biting of the stimulated paw) were recorded. The monofilament which produced nocifensive responses greater than 60% of the time was taken as the "threshold".

2.1.3 Immunohistochemistry.

Immunohistochemistry was carried out on EAE tissue taken at 3 different time points after disease induction. "Onset": lumbar spinal cords taken from mice at clinical grade 1 (n=4); "Peak": lumbar spinal cords taken from mice at clinical grade 3 or 4 (n=5); "Chronic": lumbar spinal cords taken 4 weeks after EAE induction (n=3). Lumbar spinal cords from mice that only received the CFA and pertussis treatments served as controls (n=3). Mice were anesthetized and sacrificed by transcardiac perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The lumbar enlargement of the spinal cord was removed, post-fixed for 3-4 hours

and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek® O.C.T (Optimal Cutting Temperature) compound (Fisher Scientifc, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm). The following antibodies were used: (i) rabbit anti-CGRP (1:4000, Chemicon, Temecula, CA), (ii) rabbit anti-galanin (1:4000, Chemicon, Temecula, CA), (iii) rat anti-CD3 (1:200, Serotec, Oxford, UK), (iv) rabbit anti-GFAP (1:1000, Dako, Mississauga, ON), (v) rat anti-F4/80 (1:200, Serotec, Oxford, UK). Primary antibodies were visualized using goat anti-rabbit Alexa Fluor®594 secondary antibodies (1:200, Molecular Probes, Eugene, OR).

2.1.4 Quantification of immunohistochemistry.

Images were captured with a Hamamatsu Orca ER camera (Hamamatsu Photonics, Hamamatsu, Japan) using a Leica DMI6000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). All image analysis and cell counting were carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. The innervation density of CGRP and galanin, GFAP and F4/80 in cross-sections of the lumbar spinal cord from L4 and L5 segments was measured using NIH ImageJ software. Optical density of CGRP, galanin, GFAP and F4/80 immunoreactivity was examined specifically in a standardized region that encompassed the superficial laminae (I-II) of the dorsal horn (3 sections per slide, two slides per animal). The level of background staining was determined for each section and subtracted for all optical density measurements. The number of CD3+ positive cells within the grey matter of the superficial dorsal horn (laminae I-II) was

counted in spinal segments from L4 and L5. Left and right dorsal horns were quantified and an average was calculated per section (3 sections per slide, two slides per animal).

2.1.5 Statistical analysis.

Statistical analysis was carried out using the Student's t-test and two-way repeated measures (RM) ANOVA with Tukey post hoc tests. Significance was set at $P<0.05$

2.2 Results

2.2.1 Disease progression and neurobehavioural deficits in the MOG_{35-55} model

We first assessed whether there are any observable differences in the disease course of mice with EAE induced with MOG_{35-55} from different sources. Both cohorts of mice showed characteristic clinical deficits that developed between days 13 to 19 post induction. Symptoms began as paralysis in the tail (clinical grade 1) and progressed to more severe clinical deficits corresponding to clinical grade 3 (severe hind-limb weakness with slow righting reflex) or clinical grade 4 (hind-limb paralysis in one hind-limb or both) by day 21. Overall there was a significant difference in these clinical deficits over the course of the disease between the two groups ($P=0.02$, two-way RM-ANOVA) (Fig. 2.1A). The mean day of symptom onset was slightly earlier in mice in the EAE "A" cohort (day 15 vs. 16.5 respectively) and symptom severity was greater in these mice between days 16-19 post induction

compared those in the EAE “B” cohort. While clinical deficits tended to diminish in the later stages of the disease in the EAE “B” cohort (mean clinical score of 2.1 between days 24-28), they remained severe in mice in the EAE “A” cohort during this phase of the disease (mean clinical score of 2.95 between days 24-28) ($P<0.05$, Tukey post hoc test) (Fig. 2.1A).

As an adjunct measure of neurological function we also assessed these mice using the rotorod assay. Mice were trained to walk on the rotorod at a fixed rotational speed (16rpm) prior to disease induction. All mice tested can carry out this task without any difficulty and can remain on the rotorod for the full duration of the test (180s). Impairments in gross locomotor ability can therefore be easily detected by a failure to complete this task. Performance on the rototrod test was equally affected in mice from the EAE “A” and EAE “B” cohorts compared to CFA treated controls (CFA vs. EAE “A” and CFA vs. EAE “B”; $P<0.001$, two-way RM-ANOVA). Deficits in rotorod performance emerged slightly earlier and were more severe in the EAE “A” cohort but there was no statistical difference between the overall performance of these two groups over time (EAE “A” vs. EAE “B”; $P=0.38$, two-way RM-ANOVA).

Additionally, we analyzed rotorod performance data between the EAE “A” and EAE “B” cohorts at specific clinical grades over the course of the disease. Overall, rotorod performance was not significantly different between the two cohorts of mice at any specific clinical grades ($P=0.155$, two Way RM-ANOVA) (Fig. 2.1C). Deficits in rotorod performance were however, slightly more pronounced in the EAE “A” cohort at the early stages of disease progression (clinical grade 1 and 2).

Rotorod performance became increasingly impaired as these mice progressed to more severe clinical grades 3 and 4 (Fig.2.1C). In contrast, in the EAE "B" cohort, rotorod performance was only affected at the more severe clinical grades 3 and 4 compared to their baseline activity (Fig. 2.1C). These finding demonstrate that subtle differences in neurological impairments can be detected in mice with EAE depending on the source of the antigen (i.e MOG_{35-55}) used to induce the disease. Therefore, we next asked whether these differences in disease symptoms have any effects on pain behaviours in these mice.

2.2.2 No change in the sensitivity to noxious heat in the MOG_{35-55} EAE model

We first assessed whether there were any changes in the sensitivity to noxious heat in the hindpaws of mice with EAE. Collectively, mice with EAE (both EAE "A" and EAE "B" groups) showed no significant differences in their withdrawal latencies compared to CFA controls over the majority of the testing period ($P=0.55$, two-way RM ANOVA)(Fig. 2.2A). At later stages of the disease (day 19 to 25) withdrawal latencies began to increase significantly compared to controls ($P<0.05$, Tukey post hoc test) (Fig. 2.2A). This hyposensitivity is most likely explained by the significant impairments in gross locomotor ability seen in these mice at this stage of the disease (Fig. 2.1B,C).

In addition, we analyzed how the specific cohorts of EAE mice responded in this assay at specific stages of disease progression. Responses were assessed at: "Baseline" (mean responses prior to disease induction); "Pre-symptomatic" (mean responses from the first day post induction up the first sign of clinical deficit);

"Onset" (mean responses from mice at clinical grade 1); "Peak" (mean responses from mice at clinical grades 3 and 4); and in the "Chronic" (the mean responses from mice at clinical grade 2 or lower after having reached clinical grade 3 or 4) phase of the disease. There were no significant differences in the withdrawal latencies to noxious heat between the two cohorts of EAE mice at any stage of disease progression (P=0.127, two way RM ANOVA)(Fig. 2.2B). Similarly, withdrawal latencies were not significantly affected at any stage of disease progression within each of the respective cohorts relative to their own baseline responses. These findings suggest that thermal hyperalgesia in the hindpaws is not a major symptom in the MOG_{35-55} EAE model.

2.2.3 Cold and Tactile Allodynia are the predominant sensory disturbances in the MOG_{35-55} EAE model

Cold and tactile allodynia are often-reported sensory disturbances in patients with MS (Svendsen et al., 2005). We therefore assessed whether similar symptoms can be detected in mice with MOG_{35-55} EAE. Using the acetone test to detect changes in the sensitivity to innocuous cold stimuli, we find a significant difference in the duration of response to acetone application in mice with MOG_{35-55} EAE compared to CFA treated controls over the testing period (P=0.019 two way RM ANOVA) (Fig. 2.3A). Indicative of cold allodynia, responses in EAE mice become significantly prolonged compared to controls early in the course of the disease and persist for several days until severe clinical symptoms appear (Fig 2.3A). When we assess acetone responses in each EAE cohort separately and at specific stages in the disease we

detect no significant differences in response duration between the two cohorts at any stage of the disease (P=0.141, two way RM ANOVA). Cold allodynia was equally evident in mice from the EAE "A" and "B" cohorts with significantly prolonged responses in the earliest stages of the disease at the "Pre-symptomatic" and "Onset" phases relative to their respective baseline responses (P<0.05 Tukey post hoc test) (Fig 2.3B).

Similarly, MOG_{35-55} EAE mice show a robust tactile allodynia that also becomes apparent prior to any signs of neurological deficit. Withdrawal thresholds to von Frey hair monofilaments drop significantly in mice with EAE compared to CFA treated controls beginning by day 5 post induction and persisting until major clinical deficits emerge in most mice by day 15 (P=0.004 two way RM ANOVA) (Fig 2.4A). Like cold allodynia, tactile hypersensitivity was equally evident in both cohorts of mice with EAE. There are no significant differences in withdrawal thresholds between the two cohorts at different stages of the disease (P=0.75, two way RM ANOVA)(Fig 2.4B). Mice in both the EAE "A" and "B" cohort had similar reductions in their withdrawal thresholds relative to their baseline responses at the "Pre-symptomatic" stage and at disease "Onset" (P<0.05 Tukey post hoc test). At the "Peak" stage of disease allodynia could no longer be detected and withdrawal thresholds were actually elevated compared to baseline responses in both the "A" and "B" groups (Fig. 2.4B). Again, this is most likely a reflection of the significant impairments in gross locomotor function seen in these mice that hinders proper hindlimb function and makes withdrawal responses difficult to carry out. As clinical symptoms abate in some mice in the "Chronic" phase of the disease, we find that

withdrawal thresholds tend to decrease below those seen in the baseline condition (Fig 2.4B). Taken together these findings illustrate that both cold and tactile allodynia are major sensory disturbances that can be detected early in the disease course of the MOG_{35-55} model of EAE. This behavioural hypersensitivity to both innocuous cold and tactile stimuli is independent of the specific degree of neurological deficits that arise in the disease.

2.2.4 Sensory neuropeptide expression in the spinal dorsal horn in the MOG_{35-55} EAE model

Having established that sensory processing is significantly affected in the MOG_{35-55} EAE model, we next examined potential cellular mechanisms that may underlie these changes. Since there were no significant differences in pain behaviours between the two groups, tissue from EAE "A" and "B" cohorts were pooled for this analysis. We began by examining the expression patterns of two sensory neuropeptides, calcitonin gene related peptide (CGRP) and galanin, in the superficial dorsal horn from mice with MOG_{35-55} EAE and CFA treated controls. The expression of these neuropeptides is significantly upregulated in the dorsal horn in conditions associated with neuropathic pain and allodynia (Krenz and Weaver, 1998, Bennett et al., 2000, Liu and Hokfelt, 2002, Ondarza et al., 2003). To determine whether similar changes arise in a disease model such as EAE we used immunohistochemistry and quantified the density of staining for CGRP and galanin in laminas I-II of the superficial dorsal horn of mice with EAE and CFA treated controls. Neither CGRP nor galanin staining density changed significantly with EAE

at any time point during disease progression compared to the expression seen in CFA controls (Fig. 2.5A-F)

2.2.5 Inflammation and reactive gliosis in the spinal dorsal horn in the MOG_{35-55} EAE model.

Mounting evidence suggests that inflammation and changes in glial cell reactivity in the spinal cord can significantly influence pain sensitivity in a variety of injury models (DeLeo and Yezierski, 2001, Watkins et al., 2001, Scholz and Woolf, 2007). While it is well established that the characteristic features of EAE models are inflammation and reactive gliosis in the CNS, little attention has been paid to how these reactions proceed in regions of the spinal cord dedicated to processing sensory inputs. We therefore examined the number of T cells present in the superficial dorsal horn as well as astrocyte and microglia/macrophage reactivity in this region at different stages of EAE disease progression.

Using a CD3 antibody (that detects both CD4+ and CD8+ subsets of T cells) we found significant numbers of T cells present in the superficial dorsal horn over the course of the disease (Fig 2.6B-D). In contrast there were virtually no CD3+ cells were found in the superficial dorsal horn of CFA treated control mice (Fig 2.6A). The mean number of T cells present in the superficial dorsal horn was significantly greater in EAE mice at all disease stages compared to CFA treated controls (CFA: 0.16 ± 0.1 vs. “Onset”: 87.6 ± 23.6; vs. “Peak”: 95 ± 23.3; vs. “Chronic”: 15.3 ± 3, $P<0.05$, t-test) (Fig. 2.6E).

Astrocyte reactivity was also significantly enhanced in mice with EAE compared to CFA controls (Fig. 2.7A-D). The staining density of GFAP in the superficial dorsal horn was significantly greater in EAE mice at all stages of disease progression but was most evident during the "Peak" phase of the disease ($P<0.05$, t-test) (Fig. 2.7E). Similarly, when we analyzed the expression of F4/80 to assess microglia/macrophage reactivity, we found a significant increase in staining density in this region at all stages of disease progression in EAE mice (Fig. 2.8A-E) ($P<0.05$, t-test). Unlike T cell and astrocyte responses however, enhanced microglia/macrophage reactivity was most pronounced in the "Chronic" phase of the disease (Fig. 2.8D,E). These findings suggest that, similar to other injury models associated with neuropathic pain behaviours, inflammation and reactive gliosis in the spinal dorsal horn may also be a critical mechanism underlying the behavioural hypersensitivity observed in the MOG_{35-55} EAE model.

2.3 Discussion

It is now recognized that chronic pain is a major symptom associated with MS (Clifford and Trotter, 1984, Moulin et al., 1988, Archibald et al., 1994, Svendsen et al., 2003, Osterberg et al., 2005). To date however, few studies have examined pain related behaviours in the animal model most commonly used to study the pathophysiology of MS, EAE. We have now characterized behavioural changes in sensitivity to noxious heat and mechanical stimuli as well as innocuous cold in mice with a chronic-relapsing form of EAE. Using MOG_{35-55} in female C57BL/6 mice, we demonstrate that mice with this form of EAE develop both cold and tactile allodynia

early in the disease process, prior to any overt neurological deficits, similar to some patients with MS (Osterberg et al., 2005, Svendsen et al., 2005). However, increased sensitivity to noxious heat (thermal hyperalgesia) in the hind paws does not appear to be a major symptom in this particular EAE model. At the cellular level, we have identified significant changes in glial reactivity and an increased presence of T cells in the superficial dorsal horn as potential underlying mechanisms for the allodynia observed in these mice.

Similar to MS, clinical deficits in EAE models can follow either a relapsing-remitting, or a chronic-relapsing, disease course. To date however, changes in pain sensitivity have only been examined in one type of EAE model (Aicher et al., 2004). Aicher and colleagues used $PLP_{131-155}$ to induce a relapsing-remitting form of EAE in the SJL/J strain of mice and found that mice with this form of the disease develop a thermal hyperalgesia in clinically affected body regions (i.e. the tail) that emerges at the peak of neurological deficits and persists for several weeks. In contrast, we found no significant changes in the sensitivity to noxious heat in the hindpaws of mice with MOG_{35-55} EAE. The observed differences in heat sensitivity between the $PLP_{131-155}$ model and the MOG_{35-55} EAE model used here may be accounted for by the different assays used (tail-flick vs. "Hargreaves" plantar test) or they may reflect inherent differences in heat sensitivity between the different mouse strains used in these two models (SJL/J vs. C57BL/6) (Mogil et al., 1999). However, a recent clinical report examining pain in MS has highlighted a strong association between the type and severity of pain experienced with MS subtype (Hadjimichael et al., 2007). In addition, recent data from animal studies using a viral model of MS have shown that

SJL/J mice infected with Theiler's murine encephalomyelitis virus (TMEV) exhibit distinct changes in pain sensitivity to both thermal and mechanical stimuli that are influenced by gender (Lynch et al., 2008). Therefore, it cannot be ruled out that different animal models used to study MS (i.e. $PLP_{131-155}$ EAE vs. MOG_{35-55} EAE; EAE vs. TMEV) may generate their own distinct pain "phenotype".

Interestingly, we observed differences in disease course and symptom severity in different cohorts of animals immunized with MOG_{35-55}. Even with these differences in the clinical manifestations of the disease, pain behaviours were affected equally in the two cohorts of mice. This suggests that, in the MOG_{35-55} EAE model, changes in sensory function and the development of neuropathic pain behaviours are independent of symptom severity and the clinical course of the disease.

Allodynia to cold and tactile stimuli was also evident in mice with MOG_{35-55} EAE. The most striking observation from these experiments was the development of these symptoms prior to any signs of overt neurological dysfunction. It has been noted that in a large proportion of MS patients with neuropathic pain, that pain can be a major symptom just prior to or immediately at the onset of clinical deficits in the disease (Osterberg et al., 2005). These similarities highlight the usefulness of using the MOG_{35-55} EAE model to study neuropathic pain in MS.

To assess the underlying mechanisms that mediate the development of cold and tactile allodynia in the MOG_{35-55} EAE model, we examined the innervation patterns of two sensory neuropeptides commonly associated with pain processing, CGRP and galanin, in the spinal dorsal horn. Enhanced expression or sprouting of $CGRP^+$ axons

in the dorsal horn has been identified as an important anatomic substrate for allodynia and autonomic dysreflexia in spinal cord injury models (Krenz and Weaver, 1998, Bennett et al., 2000, Ondarza et al., 2003). Similarly, neuropathic pain in peripheral nerve injury models is associated with significant alteration in the expression of galanin in the superficial dorsal horn (Liu and Hokfelt, 2002). However, in mice with MOG_{35-55} EAE we found no changes in CGRP or galanin expression in this region. Therefore, alterations in sensory processing in the MOG_{35-55} EAE model cannot be accounted for by differences in the expression patterns of these "classical" sensory neuropeptides at the spinal level.

Although we found no changes in sensory neuropeptide expression in the superficial dorsal horn we did observe significant numbers of T cells and enhanced reactivity of astrocytes and microglia/macrophages in this region at every stage of the disease ("Onset", "Peak" and "Chronic" phases). Non-neuronal cells such as microglia/macrophages, astrocytes and T cells are now recognized to be integral for the generation and maintenance of central sensitization and neuropathic pain (Watkins et al., 2001, Moalem and Tracey, 2006, Scholz and Woolf, 2007).

T cells are known to traffic into the superficial dorsal horn after peripheral nerve injury (Sweitzer et al., 2002) and mice lacking mature T cells (athymic "nude" mice) do not develop neuropathic pain after peripheral nerve injury (Moalem et al., 2004). Endoneurial T-cells have also been implicated as a potential underlying mechanism of neuritic pain (Eliav et al., 1999). In MOG_{35-55} EAE mice, CD3+ T cells are abundant in the superficial dorsal horn early in the disease correlating with development of allodynia. T cells most likely affect sensory processing through their

release of pro-inflammatory cytokines that can directly sensitize dorsal horn neurons (Kawasaki et al., 2008). There is also increasing evidence that T cells can produce and secrete neuroptophic factors such as brain derived neurotrophic factor (BDNF) (Kerschensteiner et al., 1999, Stadelmann et al., 2002). While this suggests that some infiltrating leukocytes in MS may have a neuroprotective role, BDNF is also known to modulate synaptic transmission and neuronal excitability (Kafitz et al., 1999, Lu, 2003) and is as a key mediator in the genesis of central sensitization and pain hypersensitivity (Kerr et al., 1999, Mannion et al., 1999, Coull et al., 2005, Lu et al., 2007). Whether specific subsets of T cells (i.e. CD4+ vs. CD8+; Th1 vs. Th2) differentially affect pain processing in EAE models is an important question that remains to be addressed.

Reactive gliosis in the superficial dorsal horn was also a prominent feature of mice with MOG_{35-55} EAE. Increased reactivity of astrocytes and microglia/macrophages was evident at disease onset and, particularly for microglia, remained significantly elevated into the chronic stages of the disease. Much like T cells, reactive glial cells can modulate spinal excitability and mediate pain hypersensitivity through their production and release of cytokines and growth factors such as BDNF (DeLeo and Yezierski, 2001, Coull et al., 2005). Similar to the neuropathic pain that arises as a result of peripheral nerve lesions, sensitization of spinal pain pathways and the consequent allodynia observed in the MOG_{35-55} EAE model may be triggered by increased activity in these non-neuronal cells in the superficial dorsal horn (Colburn et al., 1999, Hains and Waxman, 2006). In the case of peripheral nerve injury, it has been suggested that injury in the periphery triggers

the neuronal production of mediators such as ATP, the chemokines CCL2 (MCP-2) or fractalkine that directly activate spinal microglia (Tsuda et al., 2003, Milligan et al., 2004, Verge et al., 2004, Zhang and De Koninck, 2006). Whether similar mechanisms are engaged in EAE models needs to be determined. Given that there is little evidence for direct injury to the peripheral nervous system in the MOG_{35-55} EAE model, the most likely source of spinal glial activation in the disease is from the initial trafficking of T cells into the CNS. These early inflammatory events may then set off the cascade of responses from astrocytes and microglia that leads the observed behavioural hypersensitivities.

2.4. Conclusions

Taken together our results demonstrate that the MOG_{35-55} EAE model is a useful tool to study neuropathic pain in MS. Future studies will now be aimed at addressing the specific signaling pathways and cellular mechanisms that mediate the changes in sensory function observed in the disease. A better understanding of these mechanisms will help to tailor future therapies to treat the often-neglected symptoms of neuropathic pain in MS.

2.5 Figures

Figure 2.1

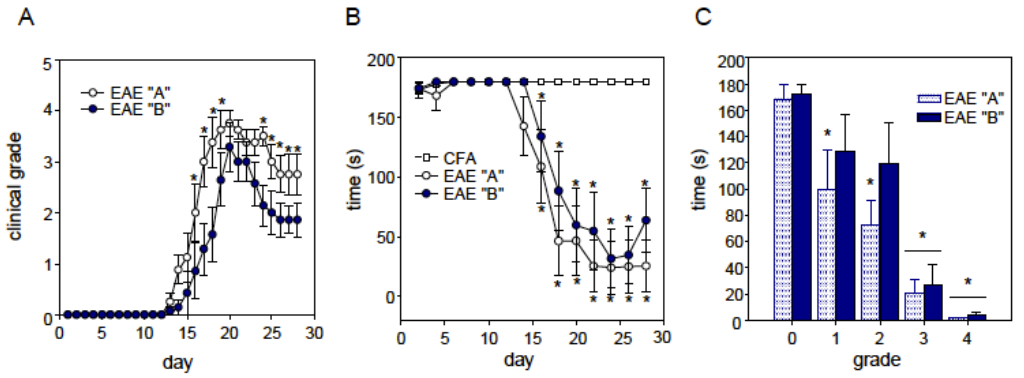

Figure 2.1
Disease progression in MOG35-55 EAE. (A) Progression of clinical deficits in the EAE "A" and EAE "B" cohorts of mice. Treatments are significantly different (*P<0.05 two way RM-ANOVA, Tukey post hoc test). (B) Rotorod performance is significantly impaired over time in mice with MOG35-55 EAE. Both cohorts are significantly impaired compared to CFA treated controls (*P<0.05, two way RM-ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice (C) Rotorod performance at specific clinical grades. Rotorod performance is significantly affected in EAE "A" mice at all stages of disease progression relative to their own baseline performance. Rotorod performance was only affected at clinical grades 3 and 4 in the EAE "B" cohort relative to baseline (*P<0.05 two way RM-ANOVA, Tukey post hoc test). There were no significant differences in overall rotorod ability between the two cohorts at any clinical grade.

Figure 2.2

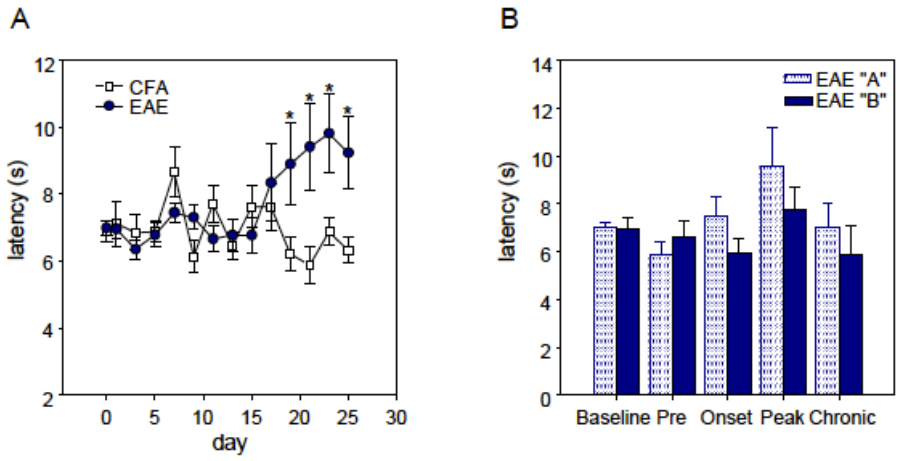

Figure 2.2
Sensitivity to noxious heat in MOG35-55 EAE. (A) Mean latency to withdraw from a noxious heat source applied to plantar surface of the hindpaw in MOG35-55 EAE mice and CFA treated controls over time. Overall there is no significant effect of group on withdrawal latencies over the testing period (p=0.55 two way RM ANOVA). Withdrawal latencies only become affected in MOG35-55 mice in the later stages of the disease (*P<0.05, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. (B) Withdrawal latencies to noxious heat between the two cohorts of EAE mice at specific stages of disease progression. There are no significant differences in responses between the two cohorts.

Figure 2.3

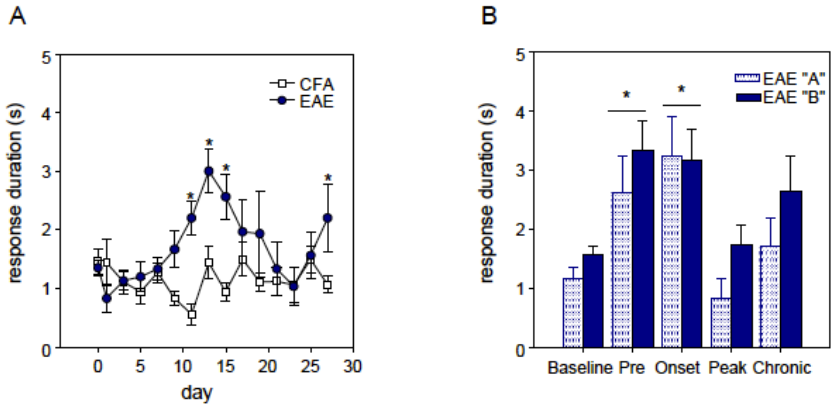

Figure 2.3
Sensitivity to innocuous cold in MOG35-55 EAE. (A) The duration of response to a single application of acetone to the hindpaw becomes significantly prolonged in MOG35-55 EAE mice compared to CFA treated controls (*P<0.05 two way RM-ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. Note that an increased duration in response is evident just prior to this period. (B) Duration of acetone responses between the two cohorts of EAE mice at specific stages of disease progression. There is no significant difference in the responses between the two cohorts at any stage. The duration of response was increased in both cohorts at the "Pre symptomatic" and "Onset" stages relative to baseline responses (*P<0.05, two way RM-ANOVA, Tukey post hoc test).

Figure 2.4

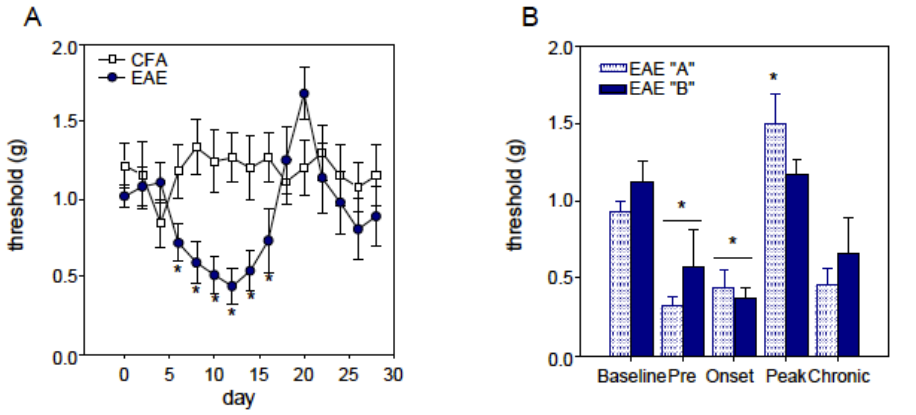

Figure 2.4
Sensitivity to punctate mechanical stimuli in MOG35-55 EAE. (A) Nociceptive withdrawal thresholds to von Frey hair stimulation are significantly reduced in MOG35-55 EAE mice compared to CFA treated controls (*P<0.05, two way RM-ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. Note the significant reduction in withdrawal thresholds prior to this period. (B) Withdrawal thresholds between the two cohorts of EAE mice at specific stages of disease progression. There are no significant differences in withdrawal thresholds between the two cohorts at any stage. Withdrawal thresholds are significantly diminished in both cohorts at the "Pre symptomatic" and "Onset" stages relative to baseline responses. Thresholds in the EAE "A" cohort are elevated relative to baseline at the "Peak" stage of disease (*P<0.05, two way RM-ANOVA, Tukey post hoc test).

Figure 2.5

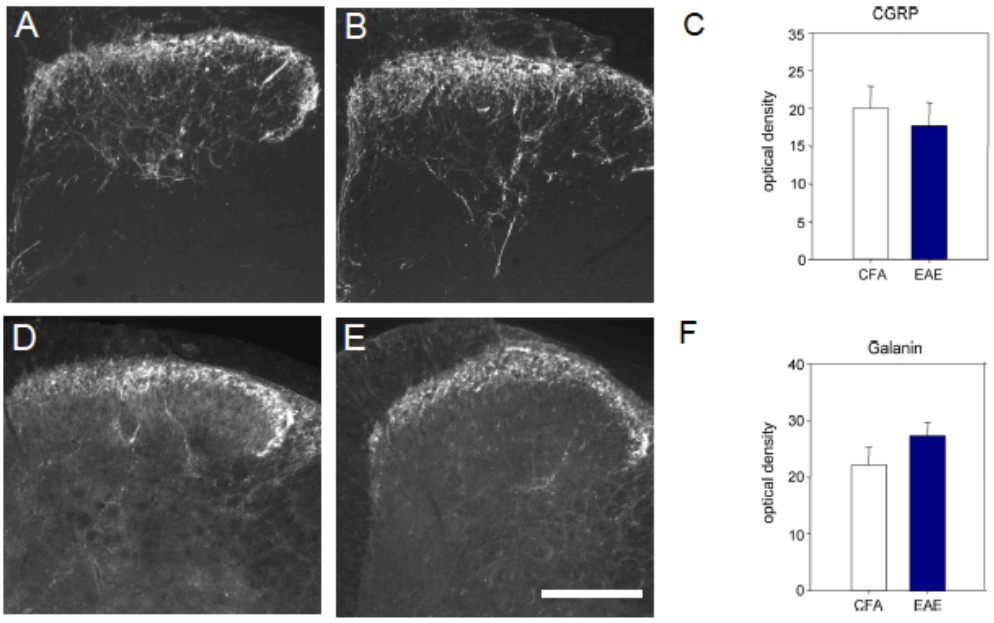

Figure 2.5
Expression of sensory neuropeptides in the superficial dorsal horn in MOG35-55 EAE. (A, B) Examples of CGRP expression in the superficial dorsal horn from a CFA treated control (A) and a MOG35-55 EAE mouse taken from the chronic phase of the disease (B). (C) There is no significant difference in CGRP expression between the two groups. (D, E) Examples of galanin expression in the superficial dorsal horn from a CFA treated control (D) and a MOG35-55 EAE mouse taken from the chronic phase of the disease (E). (F) There is no significant difference in galanin expression between the two groups. Scale bar in E=100μm and applies throughout.

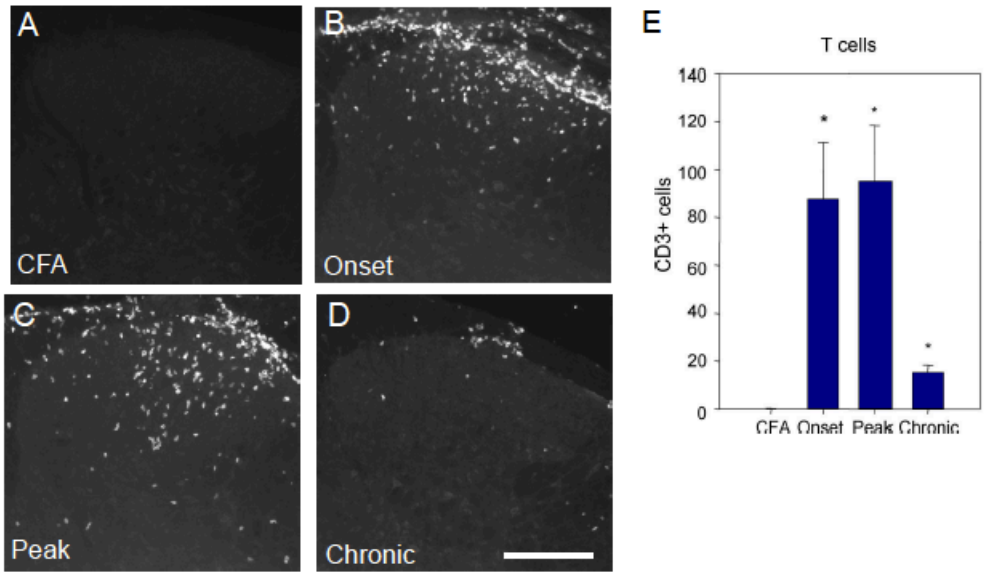

Figure 2.6
T cell expression in the superficial dorsal horn in MOG35-55 EAE. (A-D) Expression of CD3+ T cells in the superficial dorsal horn from a CFA treated control (A) and MOG35-55 EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. CD3+ cells were never observed in this region in CFA treated mice. (E) Quantification of CD3+ cells in the superficial dorsal horn. Significant numbers of CD3+ cells are found in this region at all stages of the disease compared to CFA treated controls (*P<0.05, t-test). Scale bar in D=100μm and applies throughout.

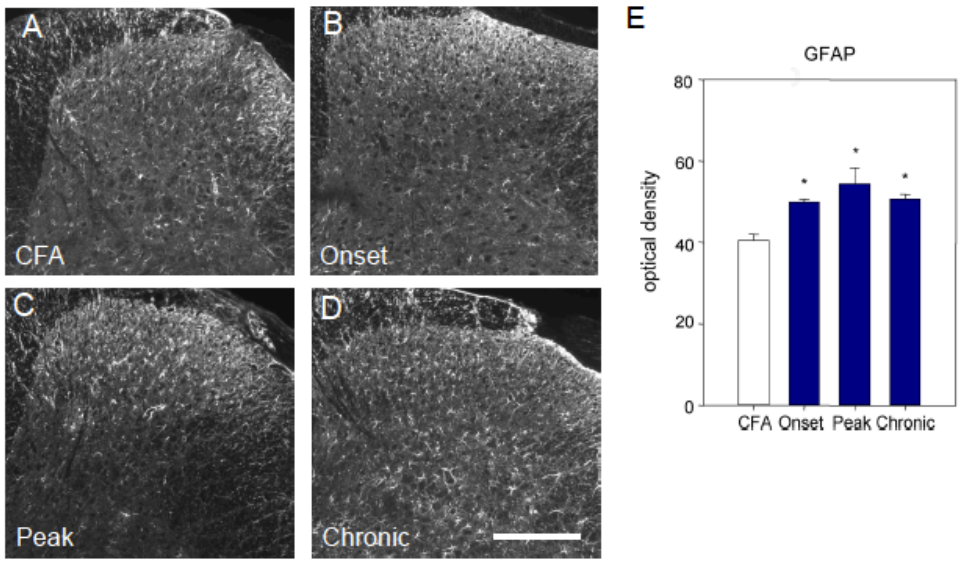

Figure 2.7
Astrocyte reactivity in the superficial dorsal horn in MOG35-55 EAE. (A-D) GFAP expression in the superficial dorsal horn from a CFA treated control (A) and MOG35-55 EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. (E) There is a slight but significant increase in GFAP expression at all stages of the disease compared to CFA treated controls (*P<0.05, t-test). Scale bar in D=100μm and applies throughout.

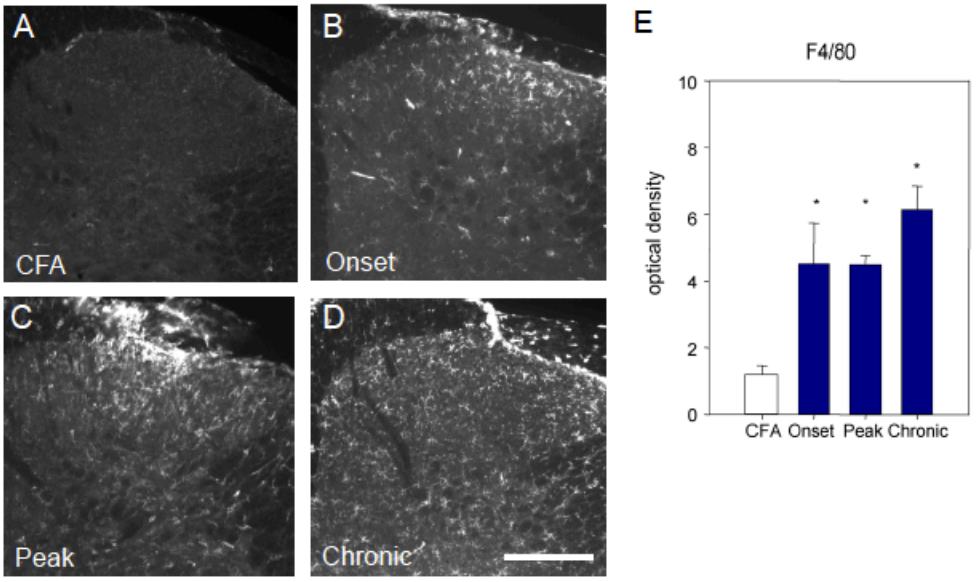

Figure 2.8
Microglia/macrophage reactivity in the superficial dorsal horn in MOG35-55 EAE. (A-D) F4/80 expression in the superficial dorsal horn from a CFA treated control (A) and MOG35-55 EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. (E) There are significant increases in F4/80 expression at all stages of the disease compared to CFA treated controls (*P<0.05, t-test). Scale bar in D=100μm and applies throughout.

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CHAPTER 3

A diminished response to formalin stimulation reveals a role for the glutamate transporters in the altered pain sensitivity of mice with experimental autoimmune encephalomyelitis (EAE)

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Pain. 2010 Jun;149(3):565-72. doi: 10.1016/j.pain.2010.03.037

3.0 Introduction

Chronic pain is now recognized as a major symptom associated with the autoimmune demyelinating disease Multiple Sclerosis (MS) (Clifford and Trotter, 1984, Archibald et al., 1994, Svendsen et al., 2003). Common pain syndromes in MS patients include trigeminal neuralgia, musculoskeletal and neuropathic pain (Moulin et al., 1988). Of these pain syndromes, neuropathic pain is the least understood and most difficult to treat (Svendsen et al., 2005). Neuropathic pain states are thought to arise from the process of central sensitization in the spinal cord (Woolf, 1983) . Central sensitization is triggered by inputs from nociceptive afferents and is associated with a reduced threshold for activation of dorsal horn neurons to noxious stimulation (Price et al., 1978).

The activation of glutamate receptors has been strongly implicated in the generation of central sensitization (Coderre and Melzack, 1992). Glutamate receptor activation can be regulated by a family of Na+dependent high affinity glutamate transporters (Danbolt, 2001, Gadea and Lopez-Colome, 2001, Huang and Bergles, 2004). In the spinal cord, three isoforms of glutamate transporters have been described (EAAT-1, EAAT-2, and EAAT-3). EAAT-1 and EAAT-2 are present in glial cells at perisynaptic sites and carry out the majority of glutamate uptake activity (Danbolt, 2001). In the spinal cord, these transporters are concentrated in the superficial dorsal horn (Tao et al., 2005) an area important for sensory input and pain processing. Dysregulation of glutamate transporter activity is associated with a persistent elevation in ambient glutamate and has been implicated as a potential

mechanism for central sensitization and neuropathic pain following peripheral nerve injury (Sung et al., 2003).

Recently, neuropathic pain behaviours have been described and characterized in two commonly used animal models to study MS, experimental autoimmune encephalomyelitis (EAE) and the Theiler's murine encephalomyelitis virus (TMEV) model (Aicher et al., 2004, Lynch et al., 2008, Olechowski et al., 2009). Mice with EAE and TMEV display characteristic neuropathic pain behaviours such as hyperalgesia to noxious heat and allodynia to cold and mechanical stimuli (Aicher et al., 2004, Lynch et al., 2008, Olechowski et al., 2008). However, the underlying mechanisms that generate neuropathic pain in these models remain unknown. In addition, it remains unclear how the responses to more persistent noxious stimuli are affected in these animals. Given that mice with EAE exhibit a robust allodynia to both cold and mechanical stimulation (Olechowski et al., 2009), we predicted that they would show a similar hypersensitivity in response to more intense noxious stimulation. To our surprise, mice with EAE showed a significant decrease in elicited pain behaviours in response to subcutaneous injection of formalin, a behavioural model of injury-induced central sensitization (Coderre et al., 1990). We show here that these effects are mediated by decreased glutamate transporter expression associated with the disease. Our findings demonstrate that dysregulation of glutamate transporter function is an important mechanism underlying the abnormal pain sensitivity of mice with EAE.

3.1 Methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with the approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta.

3.1.1 Induction of EAE.

A total of 54, 10-12 weeks old female C57BL/6 mice were used. Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. We induced EAE in mice with myelin oligodendrocyte glycoprotein 35-55 (MEVGWYRSPFSRVVHLYRNGK; MOG_{35-55}) obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB). EAE was induced by subcutaneous immunization with 50 μg of MOG_{35-55} emulsified in complete Freund's adjuvant (CFA), (Sigma-Aldrich, Oakville, ON). The CFA was supplemented with an additional 4-mg/ml heat killed M. tuberculosis H37Ra (Difco Laboratories) to a final concentration of 5mg/mL. An intraperitoneal injection of 300ng Pertussis toxin (Sigma-Aldrich, Oakville, ON) was administered at the time of induction and again 48 hours later. Control mice were treated with CFA (as above) and Pertussis toxin alone (n=28).

3.1.2 EAE Assessment

. Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1, flaccid or paralyzed tail. All experimental procedures were carried out once mice reached a score of clinical

Grade 1. We have shown previously that at this stage of the disease, mice with EAE display signs of both cold and tactile allodynia but gross locomotor behaviour is unaffected (Olechowski et al., 2009). To confirm that mice with EAE had no gross locomotor deficits that would interfere with the behavioural responses to subcutaneous formalin, they were tested on a rotorod at a fixed rotational speed of 16rpm. Mice were placed on the rotorod and the latency to fall off was determined. The maximum time for a trial was set at 180s. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. Impairments in gross locomotor function are revealed when animals can no longer remain on the rotorod for the duration of the trial.

3.1.3 Formalin test.

Prior to testing, each animal was allowed to acclimatize to the testing apparatus. The apparatus was an observation box (25cm x 23cm x 15cm) made of clear plexi-glass with a raised platform to allow for an unobstructed view of the hind paw. After acclimatization, mice were lightly restrained and given a 30μl subcutaneous injection of a 0.5% formaldehyde in saline solution into the plantar surface of one hind paw. Each mouse was then placed back into the observation box and nociceptive behaviours were monitored and timed in five-minute blocks for a thirty-minute period. Nociceptive beahaviours were defined as licking or lifting of the injected paw, flinching and vigorous shaking. The total time (in seconds) mice spent exhibiting nocicpetive behaviours was determined for the first phase (0-15 minutes) and second phase (15-30 minutes) of the response (CFA n=14; EAE n=9).

3.1.4 Drug treatments.

Cohorts of mice were treated with the drug (R)-(-)-1-methyl-1-nicotinoyl-2-pyrazoline (MS-153)(Tran and Leighton, 2006), a compound known to promote glutamate transporter activity (Shimada et al., 1999, Nakagawa et al., 2001). Mice were treated with 10mg/kg I.P. twenty minutes prior to formalin testing (see above) (CFA n=5; EAE n=6). A separate cohort of mice (CFA n=3; EAE n=3) were treated with MS-153 (10mg/kg I.P) and euthanized one hour later without any stimulation to assess FOS expression (see ***Immunohistochemistry*** below). In a separate experiment, mice (CFA n=5; EAE n=6) were treated with LY-341495 (0.25mg/kg I.P) (Tocris) a selective group II metabotropic glutamate receptor (mGluR) antagonist (Jones et al., 2005) twenty minutes prior to formalin testing.

3.1.5 Immunohistochemistry.

Immunohistochemistry was carried out on CFA or EAE mice at Clinical Grade 1. Spinal cords were taken one hour after MS-153 treatment without any further stimulation or one hour after formalin injection. Mice were euthanized with high dose pentobarbital (Euthanyl: 240mg/ml) and transcardiac perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The lumbar enlargement of the spinal cord was removed, post-fixed for 3-4 hours and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek® O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20μm). The primary

antibody used was rabbit-anti c-FOS (1:1000, Cell Signaling, Danvers, MA). The primary antibody was visualized using goat anti-rabbit Alexa Fluor®594 secondary antibodies (1:200, Molecular Probes, Eugene, OR).

3.1.6 Quantification of immunohistochemistry.

Images were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer.Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All image analysis and cell counting were carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. FOS positive cells were identified in the superficial dorsal horn (lamiae I-III). Cells were counted from dorsal horns ipsilateral and contralateral to the injection. For FOS quantification from unstimulated mice, FOS positive cells were counted from both dorsal horns, pooled and then averaged. 3 sections per slide, 2 slides per animal were analyzed.

3.1.7 Western blots.

Protein samples (20 μg) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Biorad). The membranes were blocked in 5% milk in PBS-Tween-20 (PBS-T) (0.05%), and then incubated overnight at 4 ° C in primary antibodies EAAT-1 and EAAT-2 (1: 500, Santa Cruz Biotechnology), $pNR1^{Ser896}$, $pNR1^{Ser897}$ (1:1000, Cedarlane Laboratories) and NR1 (1:1000, Sigma), diluted in PBS-T. The membranes were then washed in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti rabbit;

Jackson ImmunoResearch) diluted at 1:50,000. The membranes were washed and binding of HRP-conjugated secondary antibodies detected using chemiluminescence. (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer). Membranes were re-probed with monoclonal mouse anti-β-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

3.1.8 Statistical analysis.

Statistical analysis was carried out using the Student's t-test and one-way ANOVA with Tukey post hoc tests. Significance was set at $P<0.05$.

3.2 Results

3.2.1 Diminished pain behaviours in response to formalin stimulation in EAE mice

To determine how mice with EAE respond to a persistent noxious stimulus we assessed their behaviour in the formalin test (Coderre et al., 1993). Prior to testing, mice with EAE (clinical grade 1) were assessed for gross locomotor function using the rotorod test. No significant deficits in gross locomotor function or coordination were observed in these animals at this stage of the disease (Fig. 3.1A, also see (Olechowski et al., 2009)). Following formalin injection, mice with EAE showed significantly reduced pain behaviours when compared to the CFA control mice. Decreased nociceptive behaviours were evident in both phase 1 ($P=0.001$, t-

test)(Fig. 3.1B) and even more strikingly in phase 2 of the formalin response ($P<0.001$, t-test)(Fig. 3.1C).

3.2.2 Diminished glutamate transporter expression in EAE mice

Second phase nociceptive behaviours in the formalin test are related to activity at glutamate receptors (Yashpal et al., 2001). We therefore examined the expression of key regulators of glutamate receptor activity, the glutamate transporters (Danbolt, 2001) in mice with EAE. We began by examining glutamate transporter expression in the spinal cord of EAE mice in the absence of any noxious stimulation. Western blots determined that in the spinal cords of mice with EAE at clinical grade 1, there is a significant reduction of EAAT-1 ($P=0.001$, t-test) (Fig. 3.2A-B). There is an even more dramatic reduction in EAAT-2 expression, which is decreased to approximately 50% of the levels seen in CFA control mice ($P<0.001$, t-test) (Fig. 3.2C-D).

3.2.3 Enhanced phosphorylation of NMDA receptor subunits in spinal cords of EAE mice

Given the down regulation of glutamate transporters in EAE mice we next wanted to assess how this might affect activity at glutamate receptors. Phosphorylation of the NR1 subunit of the NMDA receptor is known to correlate with changes in nociceptive behaviours (Zou et al., 2000). In the absence of any noxious stimulation, phosphorylation of the NR1 subunit at both the serine 896 and serine 897 sites is significantly greater in the spinal cord of mice with EAE compared to CFA controls.

NR1 phosphorylation at serine 896 was found to be 72% higher in EAE spinal cords compared to CFA control mice (P<0.001, t-test)(Fig. 3.3A,D) while phosphorylation at the serine 897 site was elevated by 65% (P<0.001, t-test)(Fig. 3.3B,E). The levels of total NR1 protein were slightly reduced in the spinal cord of EAE mice but these differences were not significant (Fig. 3.3C, F).

3.2.4 Increased cellular activation in the spinal cords of EAE mice

To determine what might account for the increased phosphorylation of NMDA receptors in the unstimulated EAE spinal cord, we assessed the patterns of ongoing cellular activity in the superficial dorsal horn of mice with EAE and CFA controls. Using the protein product of the immediate early gene *c-fos* as a marker for cellular activity (Hunt et al., 1987), we find that in the absences of any noxious stimulation, FOS expression is significantly higher throughout the dorsal horn of mice with EAE compared to CFA controls (mean EAE FOS+ cells: 112 ± 9 vs. CFA FOS+ cells: 20± 6, P<0.001, one-way ANOVA, Tukey post hoc test) (Fig. 3.4A-B,E).

To establish if a loss of glutamate uptake capacity might account for this increase in cellular activation in the dorsal horn of EAE mice we assessed FOS expression after treating mice with MS-153, a compound that promotes glutamate transporter activity (Shimada et al., 1999, Nakagawa et al., 2001). In EAE mice, MS-153 treatment leads to a significant reduction in FOS positive cells in the dorsal horn compared to the untreated EAE spinal cord (EAE FOS+ cells: 112 ± 9 vs. EAE/MS153 FOS+ cells: 77 ± 8, P=0.04, one-way ANOVA, Tukey post hoc test) (Fig. 3.4C-D,E). FOS expression does however, remain significantly higher in these mice compared to

CFA controls (EAE+MS153 vs. CFA, P=0.003; EAE+MS153 vs. CFA+MS153, P=0.01, one-way ANOVA, Tukey post hoc test) (Fig. 3.4E) suggesting that additional factors also contribute to the enhanced basal activation of cells in the dorsal horn of EAE mice.

3.2.5 Cellular activation in response to formalin stimulation

We next assessed how cells in the dorsal horn of EAE mice respond to intense noxious stimulation with formalin. As expected, CFA control mice show a dramatic (approximately 4-fold) increase in the number FOS positive cells in the superficial dorsal horn ipsilateral to formalin stimulation (Fig. 3.5A, compare to Fig. 3.4A). In contrast, formalin stimulation triggers little change in the activation of cells in the dorsal horn of EAE mice. There is virtually no change in FOS expression compared to baseline levels (Fig. 3.5B, compare to Fig. 3.4B). Thus, in agreement with their behavioural phenotypes, CFA control mice are significantly more responsive at the cellular level after formalin stimulation than mice with EAE (P=0.001, t-test) (Fig. 3.5C).

3.2.6 Decreased phosphorylation of NMDA receptor subunits in the spinal cord of EAE mice in response to formalin stimulation

In the absence of stimulation, EAE mice display enhanced cellular activation and phosphorylation of NR1 subunits. Following formalin stimulation they become hyporesponsive behaviourally and have little change in their patterns of cellular activation. Therefore, we next assessed how phosphorylation of NMDA receptor subunits is affected by formalin stimulation in EAE mice. Following formalin

injection there is a significant decrease in the phosphorylation at the serine 896 site of the NR1 subunit in the spinal cords of EAE mice compared to CFA controls (P=0.035, t-test) (Fig. 3.6A,C). Phosphorylation at the serine 897 site of the NR1 subunit remains elevated in the EAE spinal cord after formalin stimulation but is no longer significantly different from the levels seen in CFA control mice (P=0.607, t test) (Fig. 3.6B and 3.6D). Taken together, these results suggest that in response to intense noxious stimulation, inhibitory signaling pathways may be engaged more readily in EAE mice.

3.2.7 Normalization of formalin responses in EAE mice

Decreased glutamate transporter expression can be associated with significant increases in ambient glutamate levels in the spinal cord (Sung et al., 2003, Liaw et al., 2005). Given the reduced levels of glutamate transporters in EAE mice, we hypothesized that following formalin stimulation, levels of extracellular glutamate might be elevated sufficiently so that presynaptic inhibitory mGluRs become activated more readily (Yamamoto et al., 2004, Jones et al., 2005). Increased activation of these inhibitory pathways in EAE mice could then account for the lack of responsiveness to formalin stimulation. To investigate this, we treated mice with an mGluR2/3 antagonist, LY-341495 prior to formalin injection. The nociceptive responses to formalin injection in EAE mice receiving LY-341495 were indistinguishable from CFA treated controls. Nociceptive behaviours were normalized in EAE mice compared to CFA controls in both phases of the formalin response (Phase 1: P=0.183, t-test)(Phase 2: P=0.387, t-test) (Fig. 3.7A-B).

To verify that these effects are mediated by a dysregulation of glutamate transporters in EAE, we assessed formalin responses after treatment with the drug MS-153. Like LY-341495, treatment with MS-153 was found to normalize the behavioural response to formalin to that of CFA controls (Phase 1: P=0.156, t-test) (Phase 2: P=0.668, t-test) (Fig. 3.7C-D). These results demonstrate that the diminished responsiveness to formalin stimulation in EAE can be accounted for by a disturbance in the glutamate uptake system.

3.3 Discussion

Previously, we have shown that mice with EAE exhibit a robust allodynia in response to acute application of innocuous cold and mechanical stimuli (Olechowski et al., 2009). We have now examined how these mice respond to a more persistent and intense noxious stimulus using the formalin model of injury-induced sensitization (Coderre et al., 1993). Unexpectedly, mice with EAE were hypo-responsive in this model, exhibiting significantly less nociceptive behaviours during both phases of the response. We show that the lack of behavioural response to formalin stimulation in these mice can be accounted for by a decreased expression of the glutamate transporters, EAAT-1 and EAAT-2 in the spinal cord. Deficits in glutamate uptake arising from the downregulation of EAAT-1 and EAAT-2 in EAE mice was predicted to allow excessive amounts of extracellular glutamate to accumulate and engage presynaptic inhibitory mGluRs more readily after formalin stimulation. By treating EAE mice with LY-341495, an antagonist for inhibitory group II mGluRs, or by promoting glutamate transporter activity with the drug MS-

153, we were able to normalize the formalin response in EAE mice. Importantly, we also show that in the absence of noxious stimulation, the spinal cords of EAE mice display signs of ongoing cellular activation and hyperexcitability that is mediated in part by the decreased levels of EAAT-1 and EAAT-2. These findings have direct implications for the underlying causes of neuropathic pain in MS.

The glutamate transporters are important for regulating the levels of extracellular glutamate at the synapse. Decreased expression of the glutamate transporters has been observed in post mortem samples from patients with MS and rats with EAE (Ohgoh et al., 2002, Vercellino et al., 2007, Pampliega et al., 2008). Glutamate transporters have also been found to be decreased in other pathologies that are associated with neuropathic pain such as after spinal cord and peripheral nerve injury (Tao et al., 2005). Spinal cord injury, MS and peripheral nerve injury are all pathologies associated with an increased inflammatory response in the CNS. Inflammation and reactive gliosis are recognized as integral processes in triggering central sensitization and neuropathic pain (Watkins et al., 2001, Moalem and Tracey, 2006, Scholz and Woolf, 2007). Inflammatory cytokines are also known to influence glutamate transporter expression *in vitro* (Tilleux and Hermans, 2007). Previously, we have demonstrated that mice with EAE show significant increases in the reactivity of microglia/macrophages along with high numbers of T-cells in the dorsal horn of the spinal cord even at the earliest stages of disease (Olechowski et al., 2009). Given that these cell types are a major source for many of the cytokines known to influence transporter expression and trafficking, it is also likely that this

early inflammatory response in the dorsal horn can account for the downregulation of EAAT-1 and EAAT-2 observed here.

A physiological consequence of decreased glutamate transporter expression in EAE mice would be an elevation in ambient extracellular glutamate levels at the synapse. Loss of these transporters could also lead to "spillover" of glutamate into nearby or adjacent synapses and the activation of distant cells. Using FOS as a marker of cellular activation, we find that, even in the absence of noxious stimulation, mice with EAE have a high level of ongoing cellular activity that is distributed throughout the dorsal horn of the spinal cord. This pattern of activity could be significantly reduced following treatment with MS-153, a compound that promotes glutamate uptake activity (Shimada et al., 1999, Nakagawa et al., 2001). While MS-153 could reduce the levels of FOS expression compared to untreated EAE mice, FOS expression was still significantly elevated in EAE mice treated with MS-153 compared to CFA controls. This suggests that additional factors also contribute to the ongoing cellular activation in the dorsal horn of EAE mice. The most likely source for these signals is the high level of reactive gliosis and inflammation found in the superficial dorsal horn of EAE mice. Reactive glia and inflammatory cells can release a number of mediators, including glutamate, which could increase the excitability and activation of neurons in the spinal cord (Watkins et al., 2001, Tilleux and Hermans, 2007). The high degree of basal activity might also account for the enhanced phosphorylation of NMDA receptor subunits seen in these mice prior to noxious stimulation. This high degree of excitability and activation suggests a likely mechanism for the hypersensitivity/allodynia observed in these animals in

response to acutely delivered innocuous stimuli (Olechowski et al., 2009). While changes in the sensitivity of peripheral nociceptors can not be excluded as a potential mechanism mediating these effects, we have previously shown that at this early stage of the disease there are no significant changes in the levels of afferent derived CGRP or galanin in the dorsal horn (Olechowski et al., 2009). It is therefore unlikely that alterations in the content of peripherally derived neuromodulators are mediating these effects.

In contrast, mice with EAE are hyporesponsive following persistent, intense noxious stimulation with formalin. Interestingly, this behavioural profile might also be accounted for by the loss of glutamate transporter expression in these mice. Studies using naïve, adult rats have shown that formalin responses can be significantly attenuated following antisense knockdown of EAAT-2 (Niederberger et al., 2003). It has been postulated that under conditions associated with a rapid increase in glutamate release from primary afferents (such as after formalin stimulation), that blockade or loss of transporter expression may result in an elevation in the levels of extracellular glutamate sufficiently so that it begins to act upon inhibitory presynaptic mGluRs (Maki et al., 1994, Niederberger et al., 2003). It is well established that agonists for pre-synaptic, inhibitory mGluR2/3 receptors can suppress formalin responses and these effects can be reversed by the antagonist LY-341495 (Simmons et al., 2002, Jones et al., 2005). In EAE mice, the loss of glutamate transporter expression coupled with the surge of afferent derived glutamate following formalin might lead to elevations in the levels of extracellular glutamate such that these inhibitory receptors are engaged more readily. These

processes would suppress glutamate release, decrease spinal activation and inhibit pain processing.

The idea that there is greater engagement of pre-synaptic inhibitory pathways in EAE mice after intense noxious stimulation is supported by our observations that the cellular activation of dorsal horn neurons in response to formalin is unaffected in mice with EAE compared to CFA controls. Additionally, formalin stimulation leads to a significant decrease in the phosphorylation of the NR1 subunit on EAE mice, particularly at the serine 896 site, suggesting that inhibitory mechanisms are being engaged under these conditions. By treating EAE mice with the selective group II mGluR antagonist, LY-341495 prior to formalin stimulation, we were able to restore formalin responses back to control levels. Furthermore, by promoting glutamate transporter activity in EAE mice with MS-153 we find a similar normalization of behaviours. This restoration of pain behaviours suggests that the initial lack of responsiveness to formalin in EAE mice does not arise due to a specific deficit in peripheral afferent input but instead highlights the role of central inhibitory mechanisms in these processes.

Alternatively, the diminished pain behaviours in EAE mice after formalin stimulation may be accounted for by a process of "reverse transport" by the glutamate transporters themselves (Phillis et al., 2000, Rossi et al., 2000). The proper functioning of the glutamate transporters is dependent on membrane potential and specific transmembrane ion gradients (Erecinska, 1989, Sarantis and Attwell, 1990). Under pathological conditions, metabolic insults can disrupt intracellular energy stores and perturb ionic gradients such that the transporters

begin to “reverse transport” glutamate out into the extracellular space (Rossi et al., 2000). Instead of absorbing excess extracellular glutamate, the transporters can become a primary source for elevating ambient glutamate levels. This phenomenon has been documented in cases of brain ischemia and in the acute phase after spinal cord injury (McAdoo et al., 2000, Rossi et al., 2000). Interestingly, it has been observed that the inflammatory insults that are the hallmarks of MS and EAE are also associated with mitochondrial dysfunction, energy failure and altered ion exchange mechanisms (Friese et al., 2007). These metabolic insults may therefore trigger a similar inverse operation of the transporters in MS and EAE. If ambient glutamate levels were elevated through these processes this could account for the allodynia and ongoing cellular activity observed in EAE mice in the absence of intense noxious stimulation. On the other hand, formalin injection itself may also be capable of triggering “reverse transport” of glutamate (Tao et al., 2005). In EAE mice, this could have an additive effect and elevate glutamate levels beyond a threshold that results in the recruitment of pre-synaptic inhibitory mGluRs and thus an attenuation of pain behaviours.

3.4 Conclusions

In summary, we now provide evidence that decreased expression of spinal glutamate transporters is an important mechanism contributing to the altered pain sensitivity of mice with EAE. Dysregulated glutamate transporter function in these mice can account for the impaired behavioural responses to formalin stimulation and also sheds light on a potential mechanism mediating the allodynia observed in this model. Given the importance of glutamate in pain processing, the glutamate

transporters may represent a novel pathway in which to manipulate activity at glutamate receptor complexes, control spinal excitability and manage neuropathic pain.

3.5 Figures

Figure 3.1

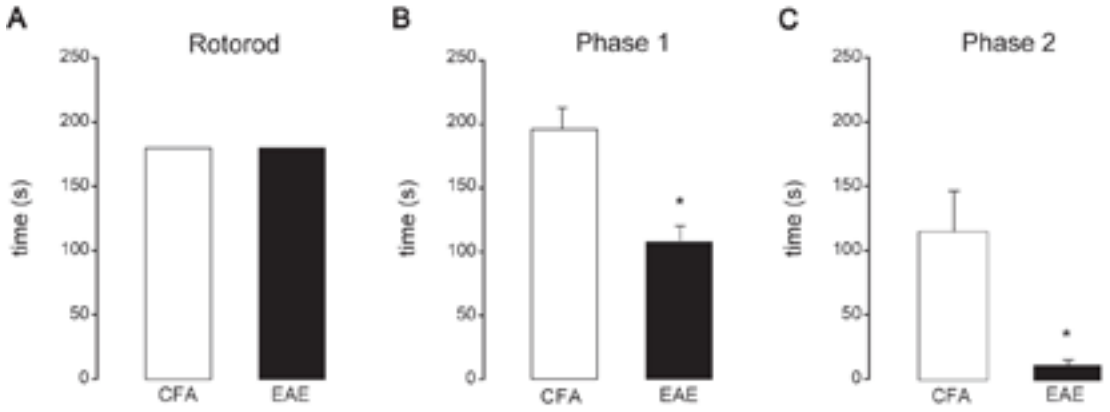

Figure 3.1
Responses to formalin injection in MOG35-55 EAE mice. (A) Gross locomotor behaviour is not affected in EAE mice at clinical grade 1. Mice with EAE show no impairments in the rotorod test at this stage of the disease. (B) Duration of nociceptive responses for phase 1 (0 to 15 minutes after formalin injection). The response to formalin is significantly decreased in EAE mice compared to CFA-treated controls (*P=0.001, t-test). (C) Duration of formalin responses in phase 2 (15 to 30 minutes after formalin injection). Responses are significantly decreased in EAE mice compared to CFA control mice (*P<0.001, t-test).

Figure 3.2

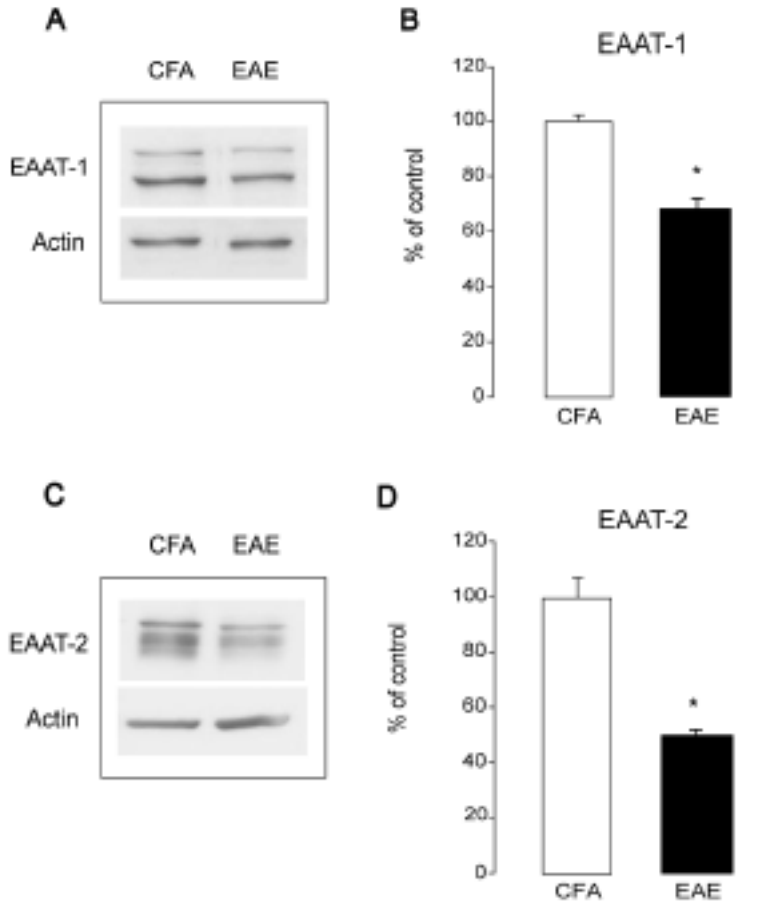

Figure 3.2
Spinal EAAT-1 and EAAT-2 expression in MOG35-55 EAE mice. (A) There is significantly diminished expression of EAAT-1 in the spinal cord of EAE mice at disease onset compared to CFA controls mice. (B) Quantification of the Western blots for EAAT-1 (*P=0.001, t-test). (C) EAAT-2 glutamate transporter expression is also significantly diminished in the spinal cord of EAE mice at disease onset when compared to CFA controls. (D) Quantification of the Western blots for EAAT-2 (*P<0.001, t-test).

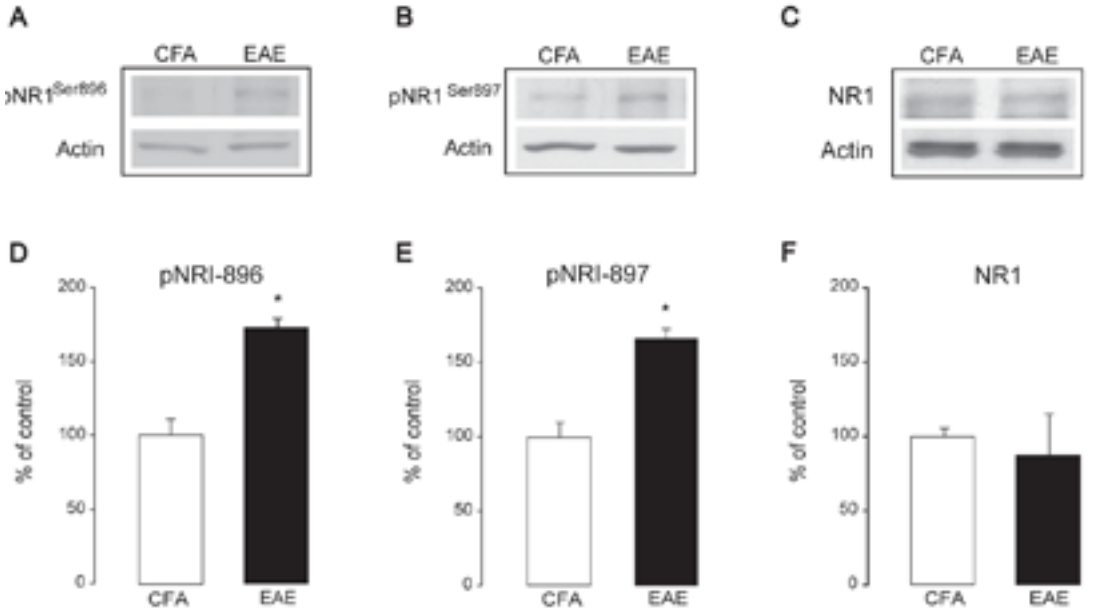

Figure 3.3
Phosphorylation of NR1 subunits in the spinal cord of MOG35-55 EAE mice. (A) There is a significant increase in the phosphorylation of NR1 at Ser896 in EAE mice at disease onset when compared to CFA controls. (B) Phosphorylation of NR1 at Ser897 is significantly increased in EAE mice at disease onset when compared to CFA controls. (C) Total NR1 expression is relatively unaffected in EAE spinal cords compared CFA controls. (D) Quantification of the Western blots for NR1Ser896 phosphorylation (*P<0.001, t-test). (E) Quantification of the Western blots for NR1Ser897 phosphorylation (*P<0.001, t-test). (F) Quantification of the Western blots for NR1. There is no significant different between the groups (P>0.05, t-test).

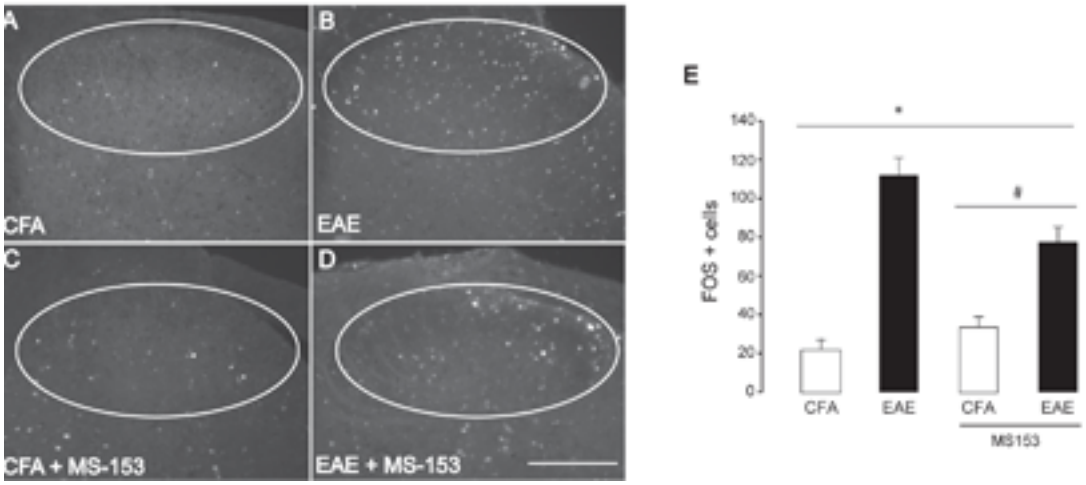

Figure 3.4
FOS expression in the spinal dorsal horn of MOG35-55 EAE mice. (A, B) Representative images of FOS expression in the superficial dorsal horn from CFA (A) and EAE (B) mice in the absence of stimulation. (C, D) Representative images of FOS expression in the superficial dorsal horn from CFA (C) and EAE (D) mice after treatment with MS-153 treatment in the absence of stimulation. (E) Quantification of FOS positive cells in the superficial dorsal horn. There is a significant increase in FOS expression in the dorsal horn of EAE mice compared to CFA controls. FOS expression is significantly decreased in EAE mice treated with MS-153 when compared to EAE mice without MS-153 treatment. FOS expression is still significantly higher in EAE+MS153 mice compared CFA controls (*P=0.01, one-way ANOVA, Tukey post hoc test, (P=0.01, one-way ANOVA, Tukey post hoc test). Scale bar in D= 200μm and applies throughout.

Figure 3.5

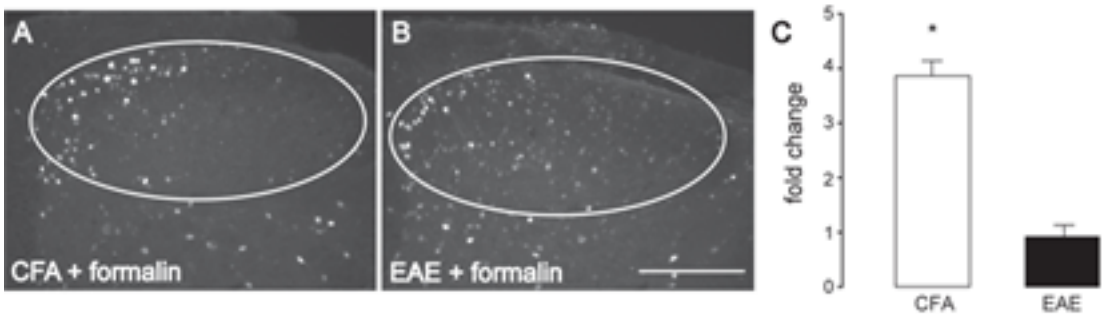

Figure 3.5
FOS expression in dorsal horn of MOG35-55 EAE mice after formalin. (A, B) Representative images of FOS expression in the ipsilateral superficial dorsal horn from CFA (A) and EAE (B) mice after formalin injection. (C) Relative to the un-stimulated condition there is a 4-fold increase in FOS expression in CFA mice after formalin injection. Relative to the un-stimulated condition, FOS expression remains unchanged in the EAE spinal cord after formalin injection (*P=0.001, t-test).

Figure 3.6

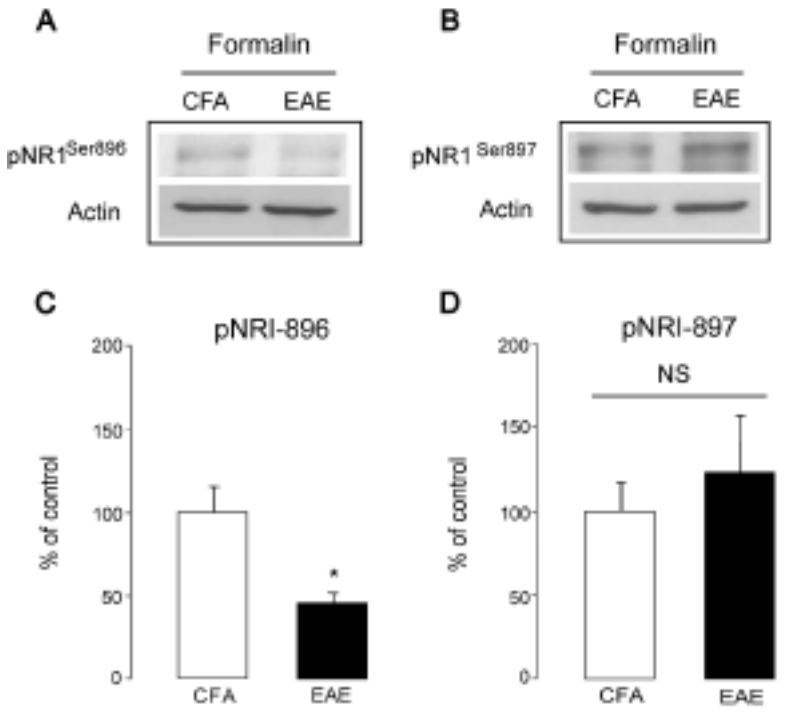

Figure 3.6
NR1 subunit phosphorylation after formalin injection. (A) There is a significant decrease in NR1Ser896 phosphorylation after formalin injection in EAE mice when compared to spinal cords from CFA controls. (B) Phosphorylation of NR1Ser897 is not significantly different between CFA and EAE spinal cords after formalin injection. (C) Quantification of the Western blots for NR1Ser896 phosphorylation (*P=0.035, t-test). (D) Quantification of the Western blots for NR1Ser897 phosphorylation (P=0.607). NS=not significant.

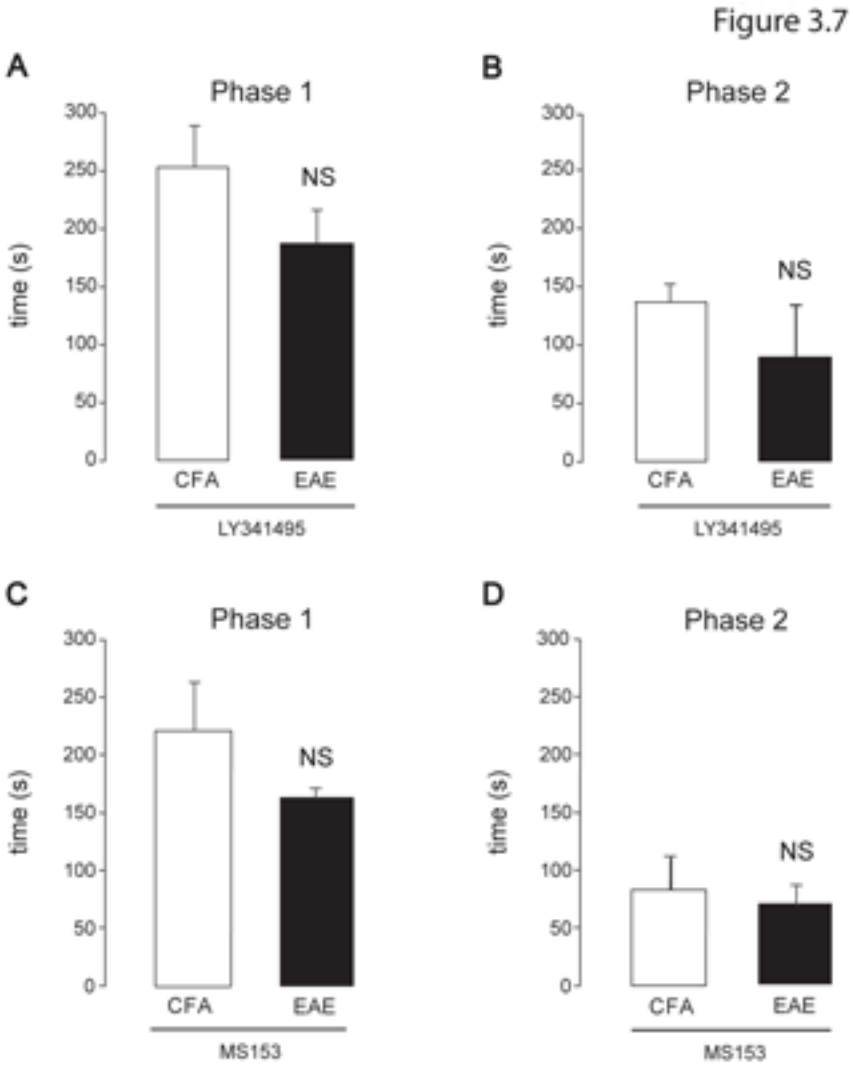

Figure 3.7
Responses to formalin injection in EAE mice after treatment with LY-341495 or MS-153. (A) Duration of nociceptive responses in Phase 1 of the formalin test after LY-341495 treatment. There is no significant difference in displayed pain behaviours between the CFA control and EAE mice (P=0.183). (B) Duration of nociceptive responses in Phase 2 of the formalin test after LY-341495 treatment. There is no significant difference in displayed pain behaviours between the two groups (P=0.387). (C) Duration of nociceptive responses in Phase 1 of the formalin test with MS-153 treatment. There is no significant difference in nociceptive responses between CFA and EAE mice (P=0.156). (D) Duration of nociceptive responses in Phase 2 of the formalin test with MS-153 treatment. There is no significant difference in nociceptive responses between CFA and EAE mice (P=0.668). NS=not significant.

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CHAPTER 4

Changes in pain sensitivity and cognitive dysfunction are independent of disease severity in experimental autoimmune encephalomyelitis (EAE)

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Introduction 4.0

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that leads to severe neurological disabilities. MS is also associated with a high incidence of cognitive impairments and neuropathic pain (Osterberg et al., 2005, He et al., 2011). Central neuropathic pain (CNP), which is thought to arise from lesions or dysfunction in the CNS, affects an estimated one third of MS patients who report pain as a major symptom of the disease(Svendsen et al., 2003, Svendsen et al., 2005). An estimated 40% to 60% of MS patients also have cognitive deficits, the most frequent being disorders of memory (He et al., 2000, Jongen et al., 2012). Disturbed memory processing can be detected in the early stages of MS and has been found to progress over time in all disease subtypes (Mandolesi et al., 2010, He et al., 2011). Although both CNP and cognitive impairments are identified in the literature as prominent symptoms reported by MS patients, little is understood about their underlying cellular mechanisms. Currently, effective treatment options are not readily available for these aspects of the disease.

The animal model experimental autoimmune encephalomyelitis (EAE) is a well-established model that has been used to study the pathophysiology of MS for over fifty years (Owens and Sriram, 1995). Despite this, it has only been recently that the model has been used to characterize changes in pain sensitivity in the disease (Musgrave et al., 2011b). Previously, we have demonstrated that neuropathic pain behaviours are present before and at disease onset in the MOG_{35-55} mouse model of EAE (Olechowski et al., 2009). We have also reported that changes

in pain sensitivity to a persistent noxious stimulus are a result of decreased expression of the EAAT-2 glutamate transporter (Olechowski et al., 2010). Changes in acute pain behaviours in an EAE rat model have also been shown to be associated with decreased glutamate transporter expression (Ramos et al., 2010). Dysregulation of glutamate transporter activity is associated with a persistent elevation in ambient glutamate and has been implicated as a potential mechanism for central sensitization and neuropathic pain following peripheral nerve injury (Sung et al., 2003).

In clinical populations, there is a strong association between chronic pain and cognitive dysfunction. However, studies using the EAE model to examine changes in cognitive function are lacking. To address this, we have carried out a series of experiments to further explore the relationship between changes in pain sensitivity and cognitive function in mice with EAE. We have monitored changes in cognitive ability using a standard behavioral assay for object recognition to determine if altered pain sensitivity is also associated with behavioral signs indicative of cognitive impairment in EAE. Using different severities of EAE we have also assessed whether changes in somatosensory and cognitive processing are dependent on the magnitude of overt clinical signs in the disease. Finally, we sought to determine whether a common underlying mechanism could mediate these changes in behavior in mice with EAE. We show here that dysregulated expression at the spinal level of the glutamate transporter subtype EAAT-2 is a common feature of EAE independent of disease severity, and promoting EAAT-2 expression using the

β-lactam antibiotic ceftriaxone (Rothstein et al., 2005) can normalize pain sensitivity and object recognition in EAE.

4.1 Methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with the approval from the Animal Care and Use Committee: Health
Sciences for the University of Alberta.

4.1.1 Induction of EAE.

A total of 58, 10–12 weeks old female C57BL/6 mice were used.
Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. We induced EAE in mice with myelin oligodendrocyte glycoprotein 35–55. (MEVGWYRSPFSRVVHLYRNGK;MOG35–55) obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB). EAE was induced by subcutaneous immunization with 50 μg of $MOG_{35–55}$ emulsified in complete Freund's adjuvant (CFA), (Sigma–Aldrich,Oakville, ON). To generate different severities of EAE the CFA was supplemented with an additional 4-mg/ml heat killed Mycobacterium tuberculosis H37Ra (Difco Laboratories) to a final concentration of 6 mg/ml. The final concentration of CFA in the emulsion was then varied across groups (EAE *Mild* CFA 0.5mg/ml; EAE *Moderate* CFA 1.5 mg/ml; EAE *Strong* CFA 3 mg/ml). An intraperitoneal (I.P.) injection of 300 ng Pertussis toxin (Sigma–Aldrich, Oakville, ON) was administered at the time of induction and again 48 h later. Control mice were treated with CFA (as above) and Pertussis toxin alone (n = 20).

4.1.2 EAE assessment.

As reported previously (Olechowski et al., 2009, Olechowski et al., 2010) Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1 (disease onset), flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both.

4.1.3 Behavioural Testing

Mechanical allodynia. A set of calibrated von Frey hair monofilaments were used to assess the sensitivity to punctate mechanical stimuli. Mice were placed in clear pexiglass chambers on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hindpaw in the ascending order of bending force (range: 0.04–2.0 g). Each hair was applied 5 times per paw, and the number of nociceptive responses (vigourous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The monofilament which produced nociceptive responses greater than 60% of the time was taken as the ‘‘threshold”.

Novel Object Recognition (NOR) Test. Mice are first presented with two identical objects (test 1) in an open field (width: 29 cm; length: 44 cm; height: 17 cm); time spent exploring each object was monitored over five minutes using a standard stopwatch. A second test (test 2) was conducted four hours later where one object was replaced with a novel object. The time spent exploring each object was

observed and recorded for five minutes. The difference in time between the two objects was calculated and then averaged for test (i.e. test 1: A-A; test 2 B-A). As a measure of total exploratory activity, the percent of time the mice spent interacting with the objects was calculated. The total amount of time spent with either of the objects over the course of the observation period in each test was divided by the total time of the observation period.

General activity/attention score. Mice were observed in the open field (width: 29 cm; length: 44 cm; height: 17 cm) for a period of 4 min. The general activity/attention score corresponds to the amount of time the mouse spent in a specific sedentary posture. The activity/attention score is a categorical score given each minute to a mouse based on the duration it spends in a specific sedentary posture. This posture is defined as having both forepaws on the ground and with the head being relatively still with a steady gaze directed below the horizontal (i.e., a head position with the nose pointing towards the floor of the observation box). As previously reported, this score was developed because, while all mice spend considerable amounts of time in one quadrant of the open field, EAE mice were often observed to adopt this posture (Musgrave et al., 2011a). These postures are never observed in naive animals and only rarely in control mice immunized with CFA alone. The total duration spent in this posture was measured using a standard stopwatch and scores were given each minute according to the following criteria: 0, mouse is still and with a floor-directed gaze for 45 or more seconds in one minute; 1, mouse is still and with a floor-directed gaze for 30–45 s in one minute; 2, mouse is still and with a floor-directed gaze for 15–30 s in one minute; 3, mouse is still and

with a floor-directed gaze for 0–15 s in one minute. These scores were totaled for each mouse at the end of the 4-min open field trial. Timing was stopped if the mouse interrupted this posture for any reason, including extensions of the head/neck forward or upward (usually to sniff at the air); large, lateral investigatory head movements, or initiation of grooming. A score of 12 is representative of maximal activity/attention during the test period.

Rotorod Assay. As a test of gross locomotor ability and coordination, mice were placed on a rotorod at a fixed rotational speed of 16 rpm. Mice were placed on the rotorod and the latency to fall off was determined. The maximum time for a trial was set at 180 s. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. After disease induction, mice were tested on alternate days. Each mouse had three trials and the mean latency to fall per trial was calculated.

4.1.4 Ceftriaxone treatment.

Cohorts of mice were treated with the β-lactam antibiotic Ceftriaxone, a compound known to be capable of upregulating glutamate transporter expression in the CNS (Rothstein et al., 2005). Mice were treated with 200 mg/kg I.P. daily starting 7 days after EAE induction (CFA n=5, EAE n=10). Vehicle controls received daily injections of saline starting at 7 days after EAE induction (CFA n=5, EAE n=10).

4.1.5 Real-time RT-PCR

RNA was isolated from spinal cords of the EAE cohorts using RNeasy Mini Kit

(Qiagen,Valencia, CA, USA) after lysis with TRIzol (Invitrogen) using the manufacturer's guidelines. RNA dissolved in deionized, diethylpyrocarbonate-treated water was used for cDNA synthesis. The primers used in real-time PCR were: EAAT2 Fwd 5'-TGA ACG AGG CCC CTG AAG AAA CTA-3' Rev 5'-ATG CCC CCG TGA ATG ATG AGG-3', IL-6 Fwd 5'-ATG GAT GCT ACC AAA CTG GAT-3' Rev 5'-TGA AGG ACT CTG GCT TTG TCT-3' IL-1β Fwd 5'-ACA GAT GAA GTG CTC CTT CCA-3' Rev 5'-GTC GGA GAT TCG TAG CTG GAT-3' GAPDH Fwd 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3' Rev 5'-CGG AGT CAA CGG ATT TGG TCG-3'. Semiquantitative analyses were performed by monitoring the increase in the fluorescence of the SYBR-green dye (Invitrogen) in real time on a Bio-Rad (Hercules, CA, USA) i-Cycler. Real-time fluorescence measurements were performed, and at threshold cycle value for each gene of interest. All data were normalized to GAPDH mRNA levels for mice.

4.1.6 Western blots.

Mice were anesthetized and euthanized by transcardiac perfusion with 0.9% saline. The brain and lumbar enlargement of the spinal cord were removed and frozen on liquid nitrogen. *Spinal Cord samples.* Protein samples (20 ul) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked in 5% milk in PBS–Tween 20 (PBS-T) (0.05%), and then incubated overnight at 4 degrees Celsius in primary antibodies EAAT-2 (1:500, Santa Cruz Biotechnology), pNR1Ser896 (1:1000, Cedarlane Laboratories) diluted in PBS-T. The membranes were then washed in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary

antibodies (goat anti-rabbit; Jackson ImmunoResearch) diluted at 1:50,000. The membranes were washed and binding of HRP-conjugated secondary antibodies detected using chemiluminescence. (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer.) Membranes were re-probed with monoclonal mouse anti-b-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

Brain Samples. Protein samples (37 ul) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Bio-Rad) overnight at 4 degrees Celsius. The membranes were blocked in 10% milk in TTBS and incubated overnight at 4 degrees in primary antibody EAAT-2 (1:1000, Santa Cruz Biotechnology) in diluted TTBS. The membranes were then washed in TTBS and incubated with HRP-conjugated secondary antibodies (goat anti-rabbit: Jackson ImmunoResearch) diluted at 1:2000. The membranes, similar to spinal cord samples, were washed and binding of HRP-conjugated secondary antibodies detected using chemiluminescence. (Amersham ECL Western Blotting Analysis System, GE Heathcare.) Membranes were re-probed with monoclonal mouse anti-β-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

4.1.7 Statistical analysis.

Statistical analysis was carried out using the Student's t-test

and one-way ANOVA with Tukey and Dunnett's post hoc tests. Significance was set at $P < 0.05$.

4.2 Results

4.2.1 Changes in pain sensitivity in EAE mice are independent of disease severity

We first assessed if there are any observable differences in the disease course of mice with EAE induced with different concentrations of Complete Freud's Adjuvant (CFA). EAE mice induced with "Moderate" CFA (CFA 1.5 mg/ml) display a more severe disease course than mice induced with "Strong" CFA (CFA 3mg/ml) and "Mild" CFA (CFA 0.5 mg/ml). EAE mice with "Moderate" CFA develop clinical signs earlier than EAE mice induced with "Mild" or "Strong" CFA and show greater overall impairments 14 days after disease induction. (Mean clinical score at day 14: EAE-Mild CFA: 0.800+/-0.389; EAE-Moderate CFA: 2.750+/-0.313; EAE-Strong CFA: 2.600+/-0.678) (Fig. 4.1A).

We have previously reported that mice with EAE develop a robust mechanical allodynia prior to and at the onset of neurological deficits (Olechowski et al., 2009). We therefore assessed whether there were any differences in the sensitivity to mechanical stimuli in the hind paws of mice with different severities of EAE. At disease onset (clinical grade 1) there was a significant decrease in the withdrawal thresholds in all three cohorts of EAE mice when compared to baseline measures (Fig. 4.1B) ($P<0.05$, one-way ANOVA, Tukey post hoc test). Decreases in withdrawal thresholds were not significantly different between the three disease

severities. Taken together, these results suggest that the changes in the sensitivity to mechanical force are independent of disease severity in EAE mice.

4.2.2 Impaired novel object recognition in EAE mice.

Deficits in cognitive function, particularly memory, are common in patients with Multiple Sclerosis (MS) (He et al., 2011). Using the Novel Object Recognition (NOR) assay as a test of cognitive ability, we next determined if similar symptoms could be detected in mice with MOG_{35-55} EAE across the different disease severities. Prior to disease induction, all mice could discriminate a novel object presented to them in the open field. In 'test 1', the mice are presented with two identical objects and the difference in time in spent with each object is around zero seconds. In 'test 2', one of the objects is replaced by a novel object of distinct shape and size and the mice spend greater time exploring and interacting with the novel object (Fig. 4.2A). Mice immunized with the CFA adjuvant alone show no impairments in the assay. For CFA treated mice, the difference in time spent with the novel object compared to the familiar object in test 2 is similar to the baseline condition (Fig. 4.2B). In contrast, novel object recognition was significantly impaired in mice with EAE at the onset of disease (clinical grade 1 for each cohort). No group of EAE mice showed signs of discrimination between the novel and familiar object in test 2 (Test 2 difference scores: Baseline: 5.1+/-0.084; CFA 7.1+/-2.45; EAE-Mild CFA: -5.343/4.343; EAE-Moderate CFA: -0.2+/-0.325; EAE-Strong CFA: -0.717/0.908)(Fig. 4.2 C, D, E).

To determine if the observed deficits in the NOR assay are due to motivational factors such as reduced overall exploratory behaviors, we calculated

the percentage of time the mice spent interacting with the objects during each test. In general, mice with EAE spend less overall time interacting and exploring the objects in Test 1 and Test 2 compared to Baseline levels and CFA treated mice (Fig. 4.2, inset graphs A-E). Taken together these findings suggest that cognitive impairments are present at disease onset in MOG_{35-55} EAE mice and similar to tactile sensitivity, are independent of disease severity.

4.2.3 Decreases in the general activity/attention score at disease onset in EAE mice

On account of the general decrease of exploratory behavior in the NOR assay, we also assessed signs of sickness behavior in EAE mice at disease onset using a general activity/attention score (Musgrave et al., 2011a). Measuring discrete postural changes can serve as adjunct measures for how the mice attend to and interact with their environment. Decreased interaction and/or attention to the surrounding environment is a characteristic feature of sickness behavior. As previously reported, prior to disease induction, healthy mice rarely score below 10–11 using this rating system, meaning that they are consistently paying attention to their surrounding environment (Musgrave et al., 2011a). At disease onset all three cohorts of EAE mice had significantly lower activity/attention scores compared to baseline measures ($p<0.001$, one-way ANOVA, Tukey post hoc) (Fig. 4.3A). Activity/attention scores were equally affected across the different cohorts of EAE mice. This suggests that a decrease in the general activity/attention score is a common feature of EAE and like pain and cognitive dyfunction, is independent of disease severity.

To determine if locomotor impairments were responsible for the decrease in exploratory behavior and the observed reductions in the general activity/attention score in mice with EAE, gross locomotor ability was assessed using a fixed-speed rotorod assay. At disease onset, when NOR assays and activity/attention were assessed, rotorod ability was not significantly affected in any cohort of EAE mice compared to CFA treated controls ($p>0.05$, one-way ANOVA) (Fig. 4.3B). The absence of gross locomotor deficits in the different cohorts of EAE mice suggests that the decrease in general activity/attention scores and reduced exploratory behavior in the NOR assay are not a consequence of impaired locomotor ability.

4.2.4 IL-6 and EAAT-2 mRNA are upregulated in all cohorts of EAE

To determine the molecular mechanisms that mediate these changes in behavior in our different cohorts of EAE mice, we began by looking at mRNA levels of the pro-inflammatory cytokines interleukin 1 beta (IL-1β), interleukin-6 (IL-6) and the glutamate transporters EAAT-2. We have previously shown that altered levels of EAAT-2 protein in the spinal cord of mice with EAE can affect pain behaviours (Olechowski et al., 2010) and other groups have implicated the involvement of glutamate transporter dysfunction in the generation of pain hypersensitivity in rat models of EAE (Ramos et al., 2010). Both IL-1β and IL-6 can regulate EAAT-2 levels (Okada et al., 2005, Sulkowski et al., 2009) and increased levels of IL-6 and EAAT-2 mRNA have been reported in other EAE models (Wang et al., 2000, Mitosek-Szewczyk et al., 2008). We found that the levels of IL-1β mRNA were elevated in EAE mice from the 'mild-*CFA*' and 'moderate-CFA' groups at disease onset but were not

significantly increased in the EAE-'strong CFA' group of EAE mice. In contrast, both IL-6 and EAAT-2 showed a consistent increase in mRNA levels across all three groups of EAE mice at disease onset when compared to CFA control spinal cord samples (Fig. 4.4A) ($p<0.05$, one way ANOVA).

4.2.5 Diminished glutamate transporter expression in the spinal cords of EAE mice at disease onset

Given the importance of the glutamate transporters (especially EAAT-2) in regulating excitatory signaling in the CNS (Danbolt, 2001), we next assessed the levels of the EAAT-2 protein in the spinal cord from the three cohorts of EAE mice using Western blots. Compared to CFA controls, all three cohorts of EAE mice had reduced levels of the major glutamate transporter EAAT-2 at disease onset **(**Fig. 4.5A). The levels of EAAT-2 were significantly less in the EAE-'moderate CFA' and EAE-'strong CFA' groups when compared to control mice treated with CFA alone ($p<0.05$, t-test) (Fig. 4.5C).

Given the deficits in the ability of EAE mice to identify a novel object in the NOR assay we next assessed the levels of EAAT-2 in the forebrain as a potential mechanism contributing to this decrease in cognitive ability. At these early stages of the disease however, we found no significant changes in the levels of EAAT-2 in the brains of mice with EAE from any cohort when compared to controls treated with CFA alone ($p>0.05$, t-test) (Fig. 4.5B, D, E). Therefore, a decrease in glutamate transporter expression in the brains of EAE mice is not contributing to the observed deficits in the NOR assay.

4.2.6 Ceftriaxone attenuates tactile hypersensitivity and cognitive deficits in EAE

To determine whether decreased levels of spinal EAAT-2 are involved in generating pain hypersensitivity and cognitive deficits in the MOG_{35-55} EAE model, we treated EAE mice with ceftriaxone, a β-lactam antibiotic that has the ability to upregulate EAAT-2 in the CNS (Rothstein et al., 2005, Ramos et al., 2010). We first assessed how ceftriaxone treatment affected the clinical signs of the disease using the EAE-'moderate CFA' protocol as this generated the most consistent disease course. Daily treatment with ceftriaxone (200mg/kg) (Melzer et al., 2008) beginning seven days after disease induction had no effect on the disease course compared to vehicle treated EAE mice (Fig. 4.6A). However, daily treatment with ceftriaxone attenuated the tactile hypersensitivity that is present at disease onset (Fig. 4.6B).

Daily ceftriaxone treatment also improved performance in the NOR assay. Prior to the start of treatment with either ceftriaxone or vehicle, all mice induced with EAE had impaired novel object recognition in test 2 compared to controls treated with CFA alone (Fig. 4.7A, ii and iii)). EAE mice then began daily treatment with either ceftriaxone or vehicle and were re-assessed in the NOR assay when the first clinical signs of the disease appeared ('onset', clinical grade 1). Object recognition remained impaired in vehicle treated EAE mice (Fig. 4.7B ii). In contrast, EAE mice treated with daily ceftriaxone had improved discrimination of the novel object in test 2 with performance approaching pre-disease levels (Fig. 4.7B iii). Interestingly, exploratory behavior remained significantly lower in both groups of

EAE mice compared to CFA controls (inset graphs Fig. 4.7B). In addition, we found that the activity/attention scores of EAE mice treated with daily ceftriaxone were not significantly different from vehicle treated controls (Fig. 4.8A). Compared to baseline values, both groups of EAE mice had significantly reduced activity/attention scores at disease onset ($p<0.001$, one way ANOVA). However, gross locomotor ability was not affected at this stage of the disease. As shown previously, rotorod performance is not significantly affected in either group of EAE mice at the onset of disease when testing takes place (Fig. 4.8B). Taken together these findings suggest that the beneficial effects of ceftriaxone in the NOR assay are not due to a generalized anti-depressant effect or through improvements in locomotor ability.

4.2.7 Ceftriaxone treatment increases EAAT-2 expression and reduces NMDA receptor phosphorylation in the spinal cord of EAE mice

To determine if ceftriaxone was increasing the levels of spinal EAAT-2 in mice with EAE, EAAT-2 levels were assessed using western blots at disease onset. The significant reduction in spinal EAAT-2 levels in vehicle treated EAE mice was prevented in mice treated with daily ceftriaxone (Fig. 4.9A,C). A consequence of dsyregulated EAAT-2 expression is an increased activation of excitatory glutamate receptors. We have previously shown that EAE mice have an increase in the levels of phosphorlyated NR1 NMDA receptor subunits at the spinal level (Olechowski et al., 2010). Increased phosphorylation of NR1 (pNR1) has been implicated in the generation of pain hypersensitivity (Olechowski et al., 2010). We therefore

examined the levels of pNR1 at serine 896 ($pNR1^{896}$) at disease onset in EAE mice treated with the vehicle or ceftriaxone. Increased levels of spinal $pNR1^{896}$ were seen in both groups of EAE mice compared to controls treated with CFA alone (Fig. 4.9B). However, $pNR1^{896}$ levels were significantly higher in EAE mice treated with vehicle compared to ceftriaxone treated EAE mice ($p<0.05$, one-way ANOVA, Tukey post hoc test)(Fig. 4.9D). These results confirm ceftriaxone's ability to upregulate or stabilize the levels of EAAT-2 in the spinal cord of the MOG_{35-55} EAE model and suggest that the decreased pain hypersensitivity and concomitant improvement in the NOR assay in ceftriaxone treated mice may arise as a result of reduced excitatory drive to spinal glutamate receptors.

4.3 Discussion

Chronic pain and cognitive deficits are now recognized as major symptoms associated with MS (Svendsen et al., 2003, Svendsen et al., 2005, Jongen et al., 2012). To date however, few studies have examined the underlying causes of these features of the disease, or the relationship between the two using the widely accepted animal model EAE,. We have previously reported that pain hypersensitivity develops in the MOG_{35-55} EAE mouse model prior to and at disease onset (Olechowski et al., 2009). We have now characterized pain sensitivity across different severities of MOG_{35-55} induced EAE. We have also examined cognitive function in this model using the novel object recognition assay across the different cohorts. Our findings demonstrate that, regardless of the severity of the clinical signs that develop, pain hypersensitivity to tactile stimuli and deficits in object recognition are consistent

features of the disease. Interestingly, normalizing pain sensitivity in mice with EAE reveals improved performance in the NOR assay, illustrating the important interrelationship between these two variables.

The development of tactile hypersensitivity across the three cohorts of EAE mice with varying severities of clinical deficits suggests that changes in pain sensitivity in MOG_{35-55} EAE are not related to the processes that lead to neurological impairment in the disease. This is similar to what is seen in patients affected with MS, where pain symptoms are not dependent on the degree of disability in a patient (Solaro and Uccelli, 2011). A common feature of both MS and the EAE models is the decrease in the protein levels of the dominant glutamate transporter EAAT-2 (Vercellino et al., 2007, Pampliega et al., 2008, Olechowski et al., 2010). Decreased levels of spinal glutamate transporters are associated with changes in nociceptive thresholds in a variety of peripheral nerve and spinal cord injury models (Tao et al., 2005). This appears to be a common feature of the MOG_{34-55} EAE model as well. Using quantitative RT-PCR, we also find that gene expression of the cytokine IL-6, is consistently upregulated in the spinal cord of EAE mice regardless of disease severity. An 'IL-6 amplifier mechanism' in the lumbar spinal cord has been characterized as a major mediator of EAE disease progression (Arima et al., 2012). Interestingly, IL-6 is also known to be a major factor triggering the downregulation of EAAT-2 (Sulkowski et al., 2009). The consistency of the observed changes in tactile sensitivity across the different cohorts of EAE mice may therefore be accounted for by these common features in EAE mice: lumbar IL-6 upregulation and subsequent decreases in the levels EAAT-2 protein in the spinal cord.

In a rat model of EAE, upregulating the levels of EAAT-2 using the β-lactam antibiotic ceftriaxone, reduces the pain hypersensitivity associated with EAE (Ramos et al., 2010). We found that in the MOG_{35-55} mouse EAE model, ceftriaxone is equally effective at normalizing tactile withdrawal thresholds. Ceftiaxone's effects appear to be mediated primarily by changes in EAAT-2 levels in the spinal cord as we found no significant changes in EAAT-2 levels in the brains of EAE mice at the early stages of disease progression when tactile hypersensitivity was most pronounced. By stabilizing EAAT-2 levels in the spinal cord, ceftriaxone treatment is also able to reduce the levels of phosphorylation at the NR1 NMDA receptor subunit, a well established pathway leading to pain hypersensitivity in a number of animal models including EAE (Zou et al., 2000).

Interestingly, we find that cognitive deficits are also consistently present in mice with MOG_{35-55} induced EAE. One of the most common cognitive deficits assessed in the MS patient population are problems with memory retention (He et al., 2011). Previous studies in both rat and mouse models of EAE reported deficits in spatial recognition using the Morris water maze (D'Intino et al., 2005, Ziehn et al., 2010). In addition, there is evidence that long-term potentiation, a cellular substrate for learning and memory, is impaired in EAE models (Mandolesi et al., 2010). Importantly, the observed deficits of mice with MOG_{35-55} EAE in the NOR assay cannot be accounted for by any significant impairment of gross locomotor ability. In all of the cohorts of EAE mice tested, we find that performance in the fixed speed rotarod assay is unchanged at the time of disease onset when NOR testing occurs. Although deficits in locomotor ability do not appear to be a factor for the impaired

performance of MOG_{35-55} EAE mice in the NOR assay, a recent study has shown anatomical evidence of neurodegeneration in the visual system of a rat model of EAE prior to the onset of optic neuritis (Fairless et al., 2012). It is unlikely however that the deficits in object recognition behaviour in MOG_{35-55} EAE mice can be accounted for by these potential early impairments of visual function as could be inferred from the latter anatomical observations of early onset retinal ganglion cell loss and microglial cell activation in this latter EAE rat model (Fairless et al., 2012). Although the timing of these neurodegenerative events in the retina coincides with the onset of the behavioural deficits reported in MOG_{35-55} EAE mice (Fairless et al., 2012) there are two critical elements that rebut this possibility: 1) we show here that treating mice with ceftriaxone can reverse the changes in NOR performance in EAE mice; and 2) retinal ganglion cell death is irreversible, and this applies to any other neurodegenerative events in the retina and optic nerve of adult mammals (Sauve and Gaillard, 1995). Therefore, it is implausible that the therapeutic effects of ceftriaxone treatment are the result of neuroprotective or regenerative responses in the retina. Furthermore, we have recently begun studies using electrophysiological parameters to assess visually-evoked retinal and cortical responses in mice with MOG_{35-55} EAE. Preliminary results from these studies indicate that there are no functional visual deficits in EAE mice at all clinical grades (from 1 to 4) compared to CFA treated control mice (B.Kerr and Y.Sauve-unpublished observations).

We did however, observe significant reductions in the total exploratory behavior of EAE mice during the NOR testing sessions. These reductions in exploration are likely related to the sickness-like behavior others and we have

documented EAE mice (Pollak et al., 2002, Pollak et al., 2003, Musgrave et al., 2011a). Decreased motivation to explore and interact with the objects due to sickness or pain is therefore more likely the cause for the poor performance in this assay. Indeed, the importance of pain hypersensitivity as a mediator of the cognitive deficits in EAE mice is highlighted by our observations that normalizing tactile withdrawal thresholds in the disease can restore object recognition without any significant change in total exploratory behavior in the test.

Taken together our results demonstrate that the MOG_{35-55} EAE model can be a useful tool for studying the mechanisms leading to pain hypersensitivity and cognitive deficits seen in MS. We show here that conserved mechanisms in EAE (increased IL-6 and concomitant reduction in EAAT-2 levels) can generate these behavioural changes independently of the pathophysiological pathways that mediate the more 'classical' neurological features of the disease such as gross locomotor impairment. Targeting the glutamate transporter system may represent an important avenue as a potential treatment option for not only abnormal pain sensitivity but also the cognitive deficits associated with the disease.

4.4 Figures

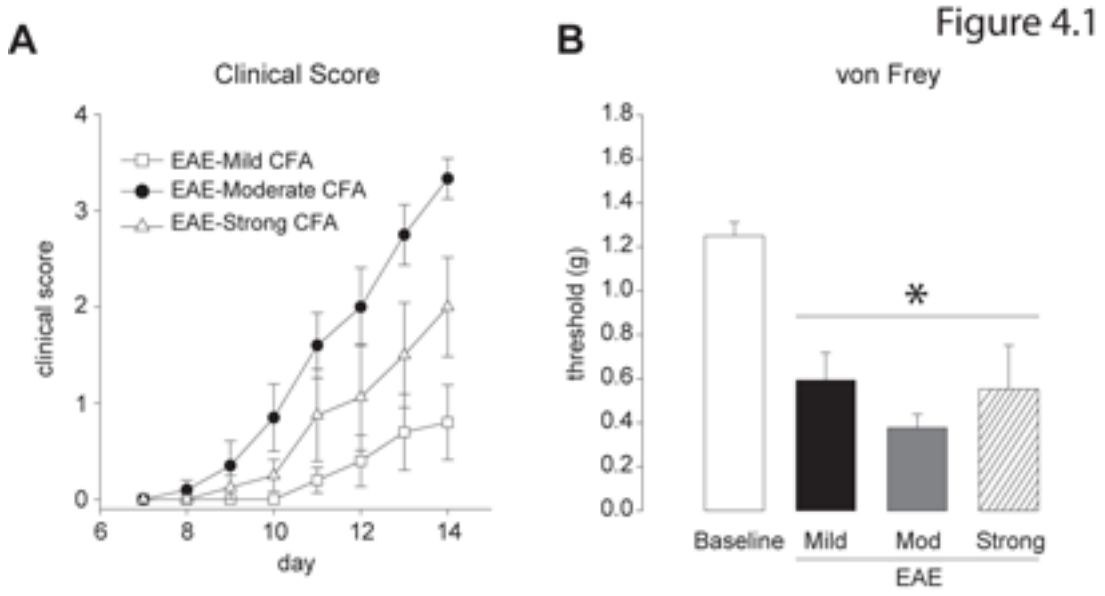

Figure 4.1
The Changes in pain sensitivity in EAE mice at disease onset are independent of disease severity (A) EAE mice induced with "Moderate" CFA (CFA 1.5mg/ml) display a more severe disease course compared to mice induced with "Strong" or "Mild" CFA ("Strong" CFA 3mg/ml or "Mild":0.5mg/ml). (B) All three groups of mice with EAE exhibit a significant increase in sensitivity to mechanical force (tactile allodynia) at disease onset when compared to baseline threshold responses (*P<0.05 one-way ANOVA, Tukey post hoc test).

Figure 4.2

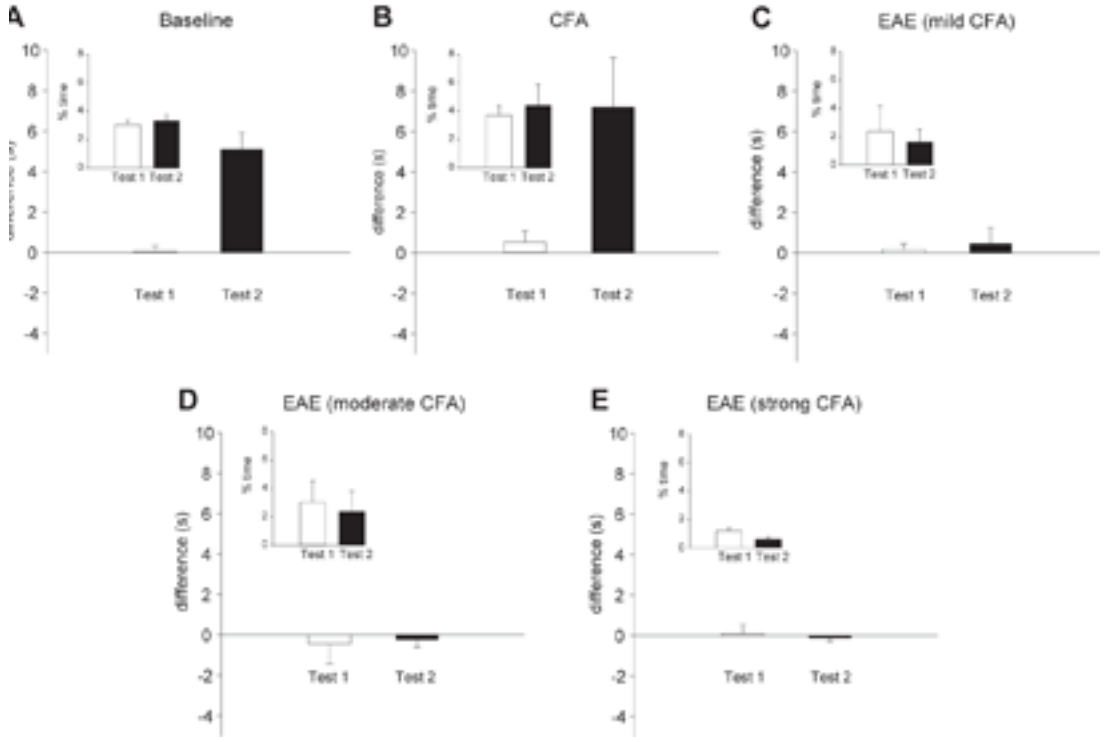

Figure 4.2
Impairment in the novel object recognition test in EAE mice. (A) All mice at baseline spend a greater amount of time with the novel object in test 2. (B) At disease onset, CFA control mice spend a greater amount of time with the novel object, similar to mice at baseline. (C-E) EAE mice with Mild, Moderate and Strong CFA do not show a preference for the novel object during test 2. Inset graphs in A-E represent the percentage of total time spent with either object during each test. This is a measure of exploratory behavior. Decreased exploratory behavior is seen in EAE mice from all three-disease severities (insets in C, D, E).

Figure 4.3

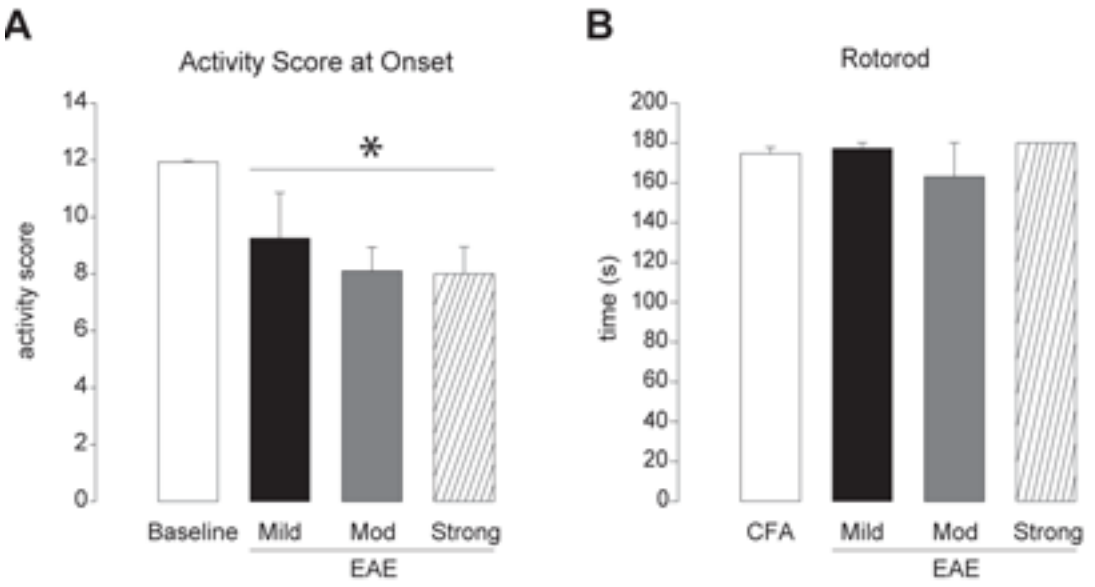

Figure 4.3
Reduced activity/attention scores in EAE mice. (A) The activity/attention score, which measures how mice attend and interact with their surroundings is Exploratory behvaiour is generally reduced in EAE mice compared to baseline measures at disease onset (clinical grade 1) in all EAE groups (*P<0.05, one-way ANOVA, Dunnett's post hoc test). (B) Gross locomotor ability in the rotorod assay at disease onset is not impaired in any cohort of EAE mice compared to CFA control mice (P>0.05, one-way ANOVA).

Figure 4.4

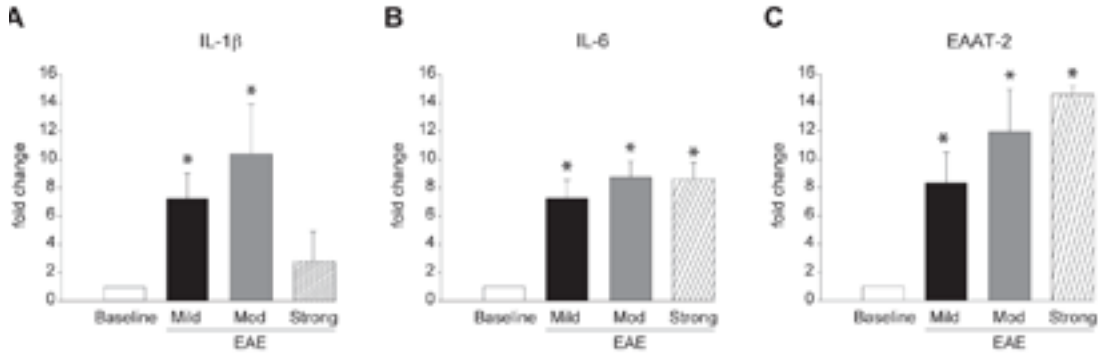

Figure 4.4
IL-6 and EAAT-2 gene expression is increased in all cohorts of EAE mice at disease onset. (A) IL-1β mRNA levels are upregulated in the spinal cords of the Mild and Moderate EAE cohorts but not in the Strong cohort. (B) IL-6 mRNA levels are upregulated in all EAE cohorts when compared to control levels. (C) EAAT-2 mRNA levels are significantly increased in all EAE cohorts when compared to CFA controls. (*P<0.05, One Way ANOVA, Dunnett's post hoc test)

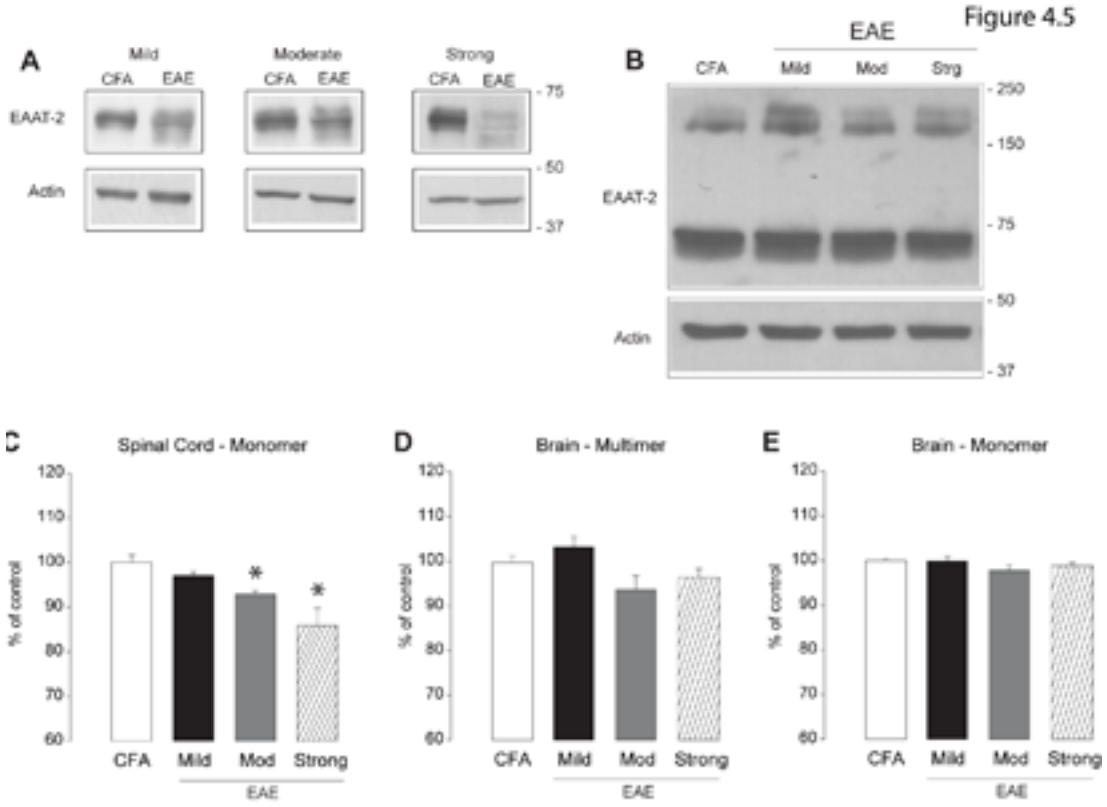

Figure 4.5
Decreased expression of the glutamate transporter EAAT-2 in spinal cords of EAE mice. (A) There is a significant decrease in the levels of spinal EAAT-2 in the Moderate and Strong EAE cohorts when compared to CFA. All samples are taken at disease 'onset' (clinical grade 1). (B) There is no difference in EAAT-2 levels (multimer or monomer) in the brains of EAE mice when compared to CFA controls. (C) Quantification of western blots from the spinal cord for EAAT-2 normalized to beta actin and expressed as a percentage of CFA control levels (*P<0.05, t-test). (D-E) Quantification of the brain EAAT-2 multimer and monomer expression in western blots normalized to beta actin and expressed as a percent of CFA control levels (P>0.05, t-test).

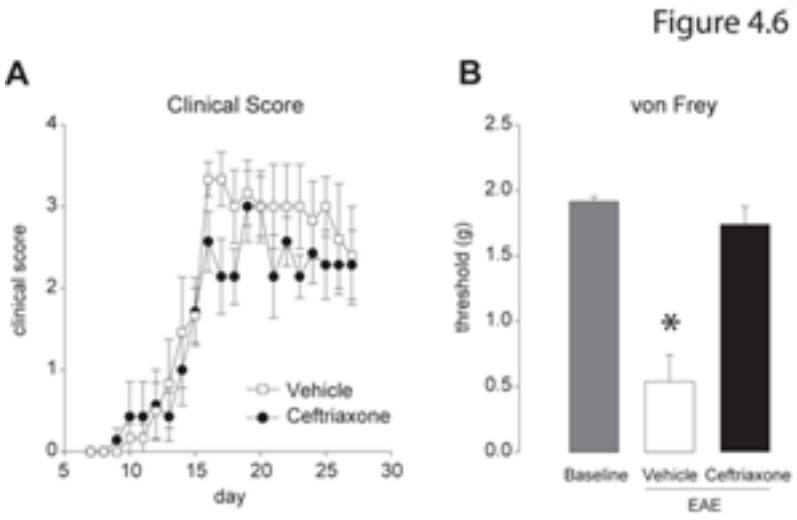

Figure 4.6
Ceftriaxone prevents tactile allodynia in EAE mice. (A) Daily treatment with ceftriaxone (200mg/kg) starting at day 7 after EAE induction does not change the disease course of EAE when compared to vehicle treated controls (EAE was generated using the Moderate CFA protocol). (B) Ceftriaxone treatment prevents tactile allodynia at disease onset (clinical grade 1) in EAE mice when compared to vehicle treated EAE mice. von Frey thresholds in EAE-vehice mice are significantly reduced compared to baseline (*P<0.05 one-way ANOVA on ranks, Dunn's post hoc test).

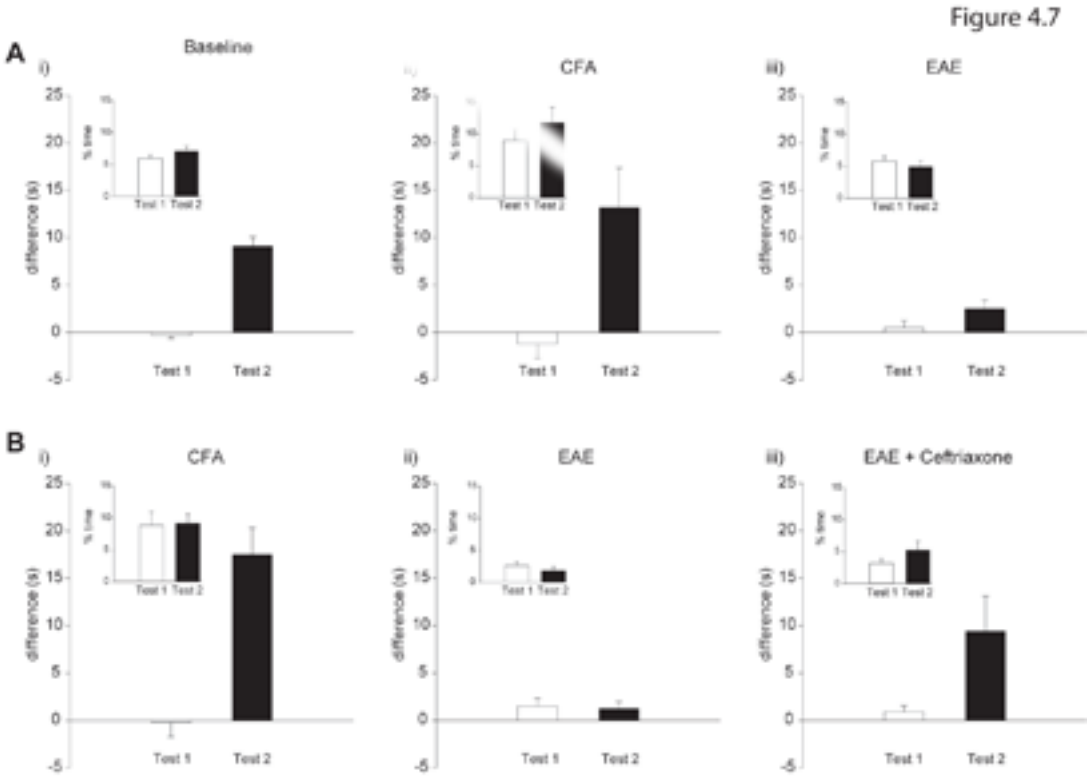

Figure 4.7
Ceftriaxone treatment restores novel object recognition in EAE mice. (A) (i) At baseline, mice spend a greater amount of time with the novel object during test 2. (ii) At day 7 after disease induction, CFA control mice spend a greater amount of time with the novel object during test 2. (iii) At day 7 after disease induction and prior to starting treatment, all EAE mice fail to show a preference for the novel object during test 2. (B) (i) At disease onset, CFA control mice spend a greater amount of time with the novel object during test 2. (ii) At disease onset, vehicle treated EAE mice continue to show impairments in their ability to distinguish the novel object during test 2. (iii) At disease onset, ceftriaxone treated EAE mice spend a greater amount of time with the novel object during test 2. Inset graphs in (A, B) represent the percentage of total time spent with the objects in each test as a measure of exploratory behavior. Exploratory behaviour remains decreased in EAE mice relative to CFA control mice regardless of treatment.

Figure 4.8

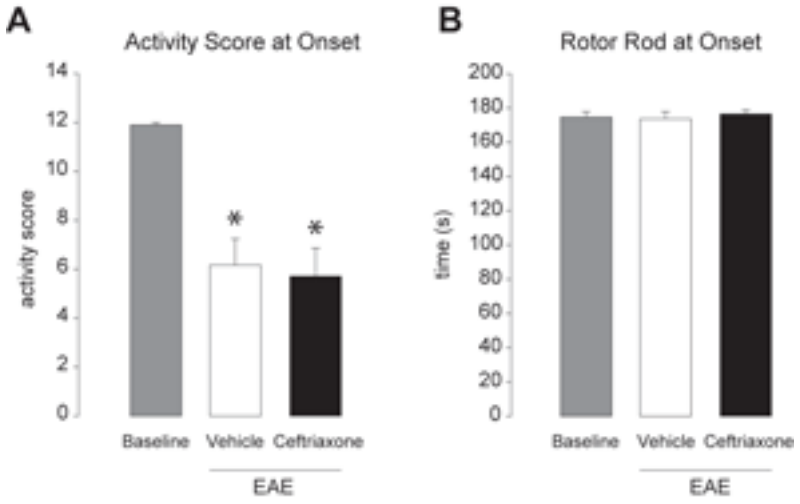

Figure 4.8
Ceftriaxone treatment does not affect activity/attention scores or gross locomotor ability at disease onset. (A) The activity/attention score is significantly decreased in both the ceftriaxone and vehicle treated EAE mice at disease onset when compared to baseline (*P<0.05, One Way ANOVA, Dunnett's post-hoc test) (B) Locomotor ability as measured with the rotorod test at disease onset is not different from baseline in both EAE groups (P>0.05, One Way ANOVA).

Figure 4.9

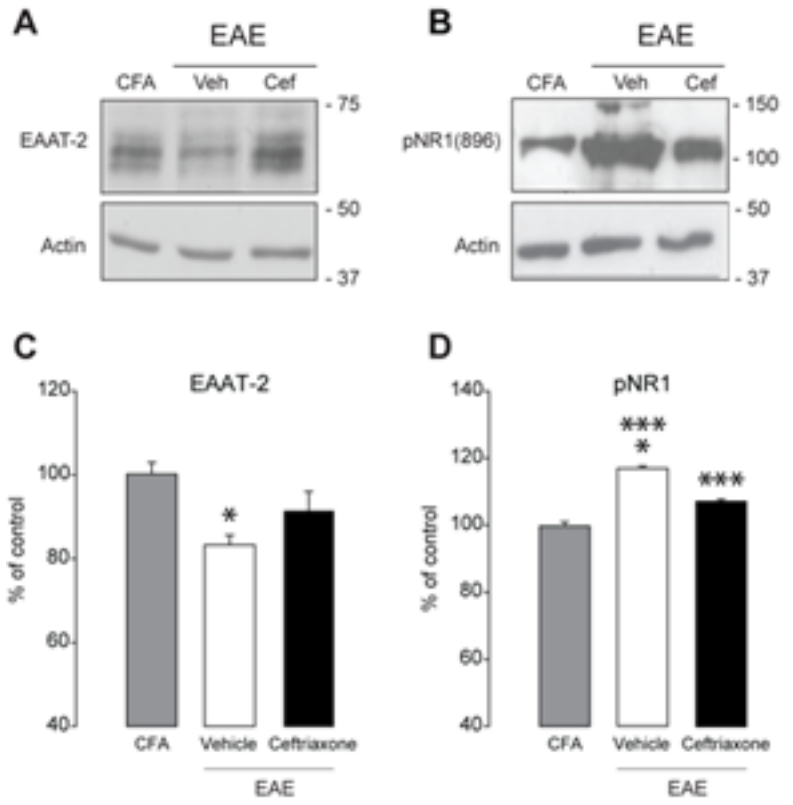

Figure 4.9
Ceftriaxone increases the levels of EAAT-2 and reduces phosphorylation of NR1 serine 896 in EAE mice spinal cords. (A) EAAT-2 levels are significantly decreased in the spinal cord of vehicle treated EAE mice at disease onset but are normalized in EAE mice treated with ceftriaxone. (B) The levels of pNR1 serine 896 are significantly higher in both groups of EAE mice compared to the levels in CFA controls. However, the levels of pNR1896 are significantly lower in the ceftriaxone treated EAE mice when compared to the vehicle treated EAE mice. (C-D) Quantification of the spinal cord Western Blots for EAAT-2 (C) and pNR1 serine 896 (D) normalized to beta actin and expressed as a percent of CFA control levels (*P<0.05, one-way ANOVA, Dunnett's post hoc test, C) (***P<0.05, one-way ANOVA, Dunnett's post hoc test, *P<0.05, t-test, EAE Vehicle vs. EAE Ceftriaxone, D).

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CHAPTER 5

General Discussion

5.0 General Discussion

Chronic pain in Multiple Sclerosis (MS) is now recognized as a major symptom of the disease (Svendsen et al., 2003, Svendsen et al., 2005). Although there are a variety of pain conditions associated with MS, neuropathic pain is the least understood and the most difficult to treat (Svendsen et al., 2005). Along with neuropathic pain symptoms, cognitive dysfunction is also identified as a major symptom of MS (Ferreira, 2010). The mechanisms behind both neuropathic pain states and cognitive dysfunction in MS are both areas where basic research and information is minimal. The current experiments focused on characterizing neuropathic pain behaviours and cognitive dysfunction in an accepted mouse model of MS MOG_{35-55} EAE. Similar to pain sensitivity (allodynia) that are reported in MS patients (Svendsen, Jensen et al. 2005), it was observed that the MOG_{35-55} animal model of EAE does result in sensory processing abnormalities just prior to and at disease onset (Chapter 2). It was also found that the MOG_{35-55} model of EAE produces cognitive deficits as indicated by the NOR test that are present just before and at disease onset (Chapter 4). The observed changes in sensory and cognitive behaviour were observed when changing the adjuvant concentrations that in turn produced three different severities of the disease, suggesting that the sensory and cognitive changes are independent of disease severity (Chapter 4). Further experiments were performed to determine the mechanisms behind the observed changes in sensory and cognitive function including investigating the role of the glutamate transporters. It was determined that a decrease in glutamate transporter activity contributes to dysfunctional sensory processing in the mouse MOG_{35-55} EAE

model (Chapter 3). Targeting and increasing the activity of glutamate transporter activity in these mice using the drug ceftriaxone resulted in the normalization of sensory and cognitive changes in this disease model at disease onset (Chapter 4). The results from these experiments validate the use of the mouse MOG_{35-55} EAE model to use for studying sensory and cognitive changes in a laboratory setting. Furthermore, the results suggest that the glutamate transporter system may be an ideal target for treatment of these changes in patients suffering from similar sensory and cognitive deficits.

5.1 Future Directions

The observations in this thesis will contribute to the understanding of the mechanisms behind sensory changes and cognitive deficits in the EAE model of MS. However, much remains to be discovered. The changes in sensory processing were characterized using classic, well-established tests of nociceptive functioning. Other groups have looked at sensory changes in the EAE model using the same tests and with similar findings in both rats and mice (Ramos et al., 2010, Thibault et al., 2011, Lu et al., 2012). The EAE disease profiles between the groups were different as a result of changes in concentration of the MOG protein used to induce the disease, anywhere between 50 to 200 μg. An increase in MOG generally results in a more severe disease profile. It was interesting to see that the disease severity did not effect the onset of allodynia in the EAE model between different studies. This supports the findings that the development of pain behaviours are independent of

disease severity within our model (Chapter 4).

Additionally some groups have reported changes in thermal sensitivity in EAE mice, although our lab was not able to replicate these findings (Aicher et al., 2004, Lu et al., 2012). One difference was that we only looked for changes in response to heat application in the hind paw, whereas other groups that found an increase in heat sensitivity reported it in the tail (Aicher et al., 2004). Recently a group published findings that in the EAE model there was onset of thermal hyperalgesia in the hindpaw over the disease course (Lu et al., 2012). When EAE was induced in SJL/J mice, hyperalegesia was present at disease onset through peak of the disease, whereas when inducing EAE in C57/BL/6 mice it was only present at the chronic phase of the disease. This is interesting because we did not pick up these changes in our C57/BL/6 mice. This could be because the furthest we followed our mice was 28 days post disease induction and this group saw the development of abnormal responses to heat application beginning at the 30 day mark. It would be interesting to follow our EAE cohorts for a longer time period to see if any changes in thermal sensitivity develop.

Changes in sensory function have been looked at in the TMEV model of MS. Onset of mechanical allodynia and onset of thermal hyperalgesia were reported. What is interesting about this study is that they found a difference in the development of pain behaviours between male and female mice (Lynch et al., 2008). The changes in sensory function in animal models of MS appear to have different profiles depending on which model is used and what type of genetic background the mice are. Further research needs to be done to characterize the sensory changes

across animal models of MS and within the EAE model itself. For example, does the sex of the animal affect the changes in sensory function observed in the EAE model similar to the TMEV model? A profile of these changes would allow researchers to chose which model and in what type of mouse they should run their experiments in depending on the specific disease profile and sensory changes they want to study.

It is interesting to see that in the EAE behavioural studies not all of the induced mice show sensory changes. Further research to determine the differences between EAE mice that show sensory changes versuses EAE mice that do not would be beneficial. For example: Is there a difference in the amount of proinflammatory cytokines or glutamate transporter expression at disease onset in the mice displaying abnormal sensory functioning? Answering these questions could help determine if there is a way to predict if an EAE mouse is going to have sensory changes and give more clues to the mechanisms behind these changes in EAE and possibly MS itself.

In recent literature the vonFrey hair and acetone tests have been criticized if they are really measuring pain behaviour and not just sensory-evoked reflex responses. One question raised is if acute pain measurements are applicable to an ongoing neuropathic pain state? (King et al., 2009). It would be interesting to determine if the same ongoing sensory changes can be measured using a more sophisticated measure of pain behaviour, such as the conditioned place aversion or preference paradigms (Sufka, 1994, Lynch et al., 2008). In these assays the affective dimension of pain (unpleasantness) is tested as opposed to just the sensory dimension of pain. Through the use of negative reinforcement (pain relief e.g

through treatment) in different contextual cues (e.g. location) the animal's subsequent behaviour can help determine if a pain treatment is effective on the affective dimension and not just the sensory dimension of pain. In this same vain we only used one test as a measure for cognitive dysfunction in this model. Future experiments using different tests of cognitive function, such as the Morris water maze or the Y-maze to determine if other types of memory or learning are affected in this animal model would also be beneficial. It would also be interesting to test changes in cognitive function at different time points of the disease course, for example at peak or chronic phases of the disease to see if the cognitive impairments are more pronounced as the disease course progresses. Additionally, it would be interesting to see if ceftriaxone treatment had any effects in these assays.

Another behavioural change that requires further exploration is the change in open field behaviours that were observed. Of specific interest is the change in activity score observed in the EAE mice just prior too and at onset of disease symptoms (Chapter 4). There is a significant decrease in activity or exploration of the open field environment during these two time points. The open field test measures spontaneous locomotor activity and it has been validated that anxiety causes mice to display thigmotaxis behaviour (tendency to stay in the periphery of the open field container). Other groups have reported a similar decrease in activity in the open field test over the EAE disease course in this model and evidence of anxiety using different tests e.g. light-dark-box (Peruga et al., 2011, Haji et al., 2012). One group suggested that the observed increase of pro-inflammatory cytokines TNF-α and IL-6 are associated with increased anxiety-like behaviour in EAE,

although the observations were all done during the chronic phase of the disease (Peruga et al., 2011). Similarly, another group looking at anxiety like behaviours at earlier time points also related the onset of symptoms of anxiety to TNF-α (Haji et al., 2012). It would be interesting to see if targeting the glutamate transporters at any of these time points would influence the onset of anxiety like behaviours. A strong link between an increase in an inflammatory environment and the onset of anxiety, cognitive deficits and pain has been reported across many different disorders (Dantzer, 2001, Musgrave et al., 2011b). In fact human MS patients commonly report these symptoms in conjunction. It would be interesting to use the EAE model to try and tease out if there is an underlying mechanism behind these behaviours in addition to the inflammatory component. For example: do the glutamate transporters play a role? The fact that the EAE model displays all of these so-called sickness behaviours makes it an excellent candidate to use for further research into this common profile of symptoms witnessed across inflammatory disorders.

The experiments using the ceftriaxone drug treatment were based on a treatment schedule from previous literature starting at day 7 after disease induction. Additional experiments to determine if treatment with a drug targeting the transporter system at the first signs of disease onset (grade 1) would be more applicable to the MS patient population. It would also be intriguing to study EAE in glutamate transporter knock out mice. EAAT-2 knock out mice have been generated, unfortunately this knock out results in lethal spontaneous seizures and motor disturbances which would make it difficult to do beahvioural assays (Maragakis and

Rothstein, 2001). Using transgenic glutamate transporter reporter mice e.g. GFP reporter would be interesting to study the changes in transporter regulation over the entire EAE disease course (Rothstein et al., 2005, Gincel et al., 2007). This technique could help determine the best time to start treatment with drugs that target the transporter system by indicating at what point after disease induction transporter regulation begins to be disrupted.

Finally, the tissue analysis done in conjunction with the behavioural experiments focused on changes of different cellular mechanisms including transporter expression in the spinal cord. Further experiments to determine what changes are happening at higher processing centers such as the brainstem and brains of the affected mice would be beneficial. This would help contribute to an understanding of the underlying mechanisms of changes that are happening throughout the entire CNS of these animals. We did quantify changes in the EAAT-2 protein in the brains of our EAE mice and did not find a significant decrease in the protein expression when compared to our control mice (Chapter 4). We only analyzed gross brain tissue, which may have confounded our results because there was just too much protein in our samples to pick up any changes in transporter expression. It would be interesting to look for changes in specific brain areas related to sensory processing and cognitive process for example, the hippocampus to see if we can find any changes in glutamate transporter expression.

Our laboratory has found a characteristic change in different amino acids throughout the disease course in both the brain and spinal cord in the model of EAE we use (Musgrave et al., 2011a, Musgrave et al., 2011c). Do these changes in amino

acid levels play a role in the change in activity of glutamate transporters in EAE is another question that needs to be addressed. Determining these mechanistic changes would be beneficial to understand the changes in pain and cognitive processing related to MS. Similar mechanisms may also play a role in other diseases where pain processing and cognitive deficits are present.

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Appendix (published papers)

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Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE)

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). While the primary symptoms of MS are losses of sensory and motor functions, it is now recognized that chronic pain is also a major concern affecting between 50% and 80% of MS patients. To date, however, few studies have examined the underlying mechanisms of chronic pain in MS or in the animal model, experimental autoimmune encephalomyelitis (EAE), which shares many features of MS pathology. We, therefore, set out to characterize the changes in pain sensitivity that arises in a chronic-relapsing model of EAE. We show here that female C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG_{35-55}) develop a robust allodynia to both cold and tactile stimuli. Allodynia emerges early in the disease process, often before any signs of neurological deficit and is independent of the overall symptom severity in these mice. "Classical" cellular substrates for neuropathic pain and allodynia such as altered expression of sensory neuropeptides in the dorsal horn of the spinal do not appear to underlie these changes in sensory function. There is, however, a significant influx of CD3+ T cells and increased astrocyte and microglia/macrophage reactivity in the superficial dorsal horn of mice with MOG_{35-55} EAE. This suggests that inflammation and reactive gliosis may be key mediators of allodynia in MOG_{35-55} EAE similar to peripheral nerve and spinal cord injury models. Taken together, our results show that the MOG_{35-55} EAE model is a useful tool to study neuropathic pain in MS.

1. Introduction

Chronic pain is a common, but little understood symptom associated with the autoimmune disease Multiple Sclerosis (MS) [2,6,29,37]. It has been estimated that approximately 50–80% of MS sufferers experience clinically significant pain at some point in the course of their disease [31]. Neuropathic pain, which arises due to lesions or dysfunction in the central nervous system (CNS), is the most prevalent and difficult to treat pain syndrome seen in MS patients [36]. However, studies focused on identifying the underlying mechanisms of neuropathic pain associated with MS are lacking.

Experimental autoimmune encephalomyelitis (EAE) is an accepted animal model of MS that shares many features of the pathology seen in MS patients, including widespread CNS inflammation, demyelination and locomotor impairments [3,32,35]. EAE can be induced in genetically susceptible mouse strains by immunization with small fragments of myelin antigens such as myelin basic protein 84–104 (MBP_{84-104}); proteolipid protein 139–151 ($PLP_{139-151}$); or myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) [5]. Depending on the strain of mouse and myelin antigen used, different disease courses can be modeled that reflect the differences in symptom progression often observed in human MS. While EAE has been used effectively to study the classical neurological deficits associated with MS such as locomotor dysfunction and paralysis, there is limited data on the specific changes in somatosensory processing that occur in the model.

A recent study has described altered pain behaviours in a relapsing–remitting form of EAE in the SJL/J strain of mice after immunization with $PLP_{131-151}$ [1]. These mice show signs of thermal hyperalgesia (an increased sensitivity to noxious heat) that develops at the peak of neurological deficits and persists throughout the course of the disease [1]. While these findings are an important validation of using the EAE model to study MS-related pain, only one sensory parameter (i.e., hyperalgesia) has been published thus far. Cold and tactile allodynia (painful responses to non-painful stimuli) are also often reported from MS patients with neuropathic pain [31,36].

To address this, we have used a chronic-relapsing model of EAE in female C57BL/6 mice immunized with MOG_{35-55} and assessed changes in sensitivity to noxious heat, innocuous cold and

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mechanical stimuli. In addition, because inflammatory processes and reactive gliosis in the spinal cord are known to contribute to neuropathic pain behaviours in peripheral nerve and spinal cord injury models [7,9,12,39] we have also examined these responses in the superficial dorsal horn in mice with MOG_{35-55} EAE.

2. Materials and methods

2.1. Induction of EAE

A total of 39, 10–12 weeks old female C57BL/6 mice were used. Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. All animal procedures and experiments were approved by the University of Alberta Health Sciences Laboratory Animal Services. The course and severity of symptoms in EAE are often variable between different laboratories even when using the same antigen, doses and induction protocols. Although a number of factors can account for these variations, differences in the source of where MOG_{35-55} is obtained from can be a major factor. Therefore, we induced EAE in mice with myelin oligodendrocyte glycoprotein 35–55 (MEVGWYRSPFSRVVHLYRNGK; MOG_{35-55}) obtained from two different sources to determine whether this would generate differences in disease course and symptom severity and if so, if this has any effect on pain behaviours. MOG_{35-55} peptide was obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB) (a generous gift from Dr. Chris Power, University of Alberta) and also from AnaSpec Inc (San Jose, CA). EAE was induced by subcutaneous immunization with 50 μg of MOG_{35-55} from either source (grouped as EAE "A" $n = 10$ or EAE "B" $n = 10$, respectively) emulsified in complete Freund's adjuvant (CFA) (1 mg/ml to a final concentration of 0.5 mg/ml CFA, Sigma–Aldrich, Oakville, ON). An intraperitoneal injection of 300 ng Pertussis toxin (Sigma–Aldrich, Oakville, ON) was administered at the time of induction and again 48 h later. Control mice were treated with CFA (0.5 mg/ml) and Pertussis toxin alone ($n = 10$).

2.1.1. EAE assessment

Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1, flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one hindlimb or both [15].

2.2. Behavioural testing

All behavioural testing was carried out by an experimenter blinded to the specific treatment groups. Mice were given 15–20 min to acclimatize to the testing apparatus for a given assay before the testing began.

2.2.1. Rotorod test

As a test of gross locomotor ability and coordination, mice were placed on a rotorod at a fixed rotational speed of 16 rpm. Mice were placed on the rotorod and the latency to falloff was determined. The maximum time for a trial was set at 180 s. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. After disease induction, mice were tested on alternate days. Each mouse had three trials and the mean latency to fall per trial was calculated.

2.2.2. Thermal hyperalgesia

After disease induction, mice were tested on alternate days using the Hargreaves test [13]. This test measures the latency to withdrawal from a noxious heat source applied to the plantar surface of the hindpaws. To prevent tissue damage, a cutoff score of 20 s of applied heat was used. Withdrawal latencies were recorded three separate times for each paw, and the average withdrawal latency was calculated.

2.2.3. Cold allodynia

A single drop of acetone was applied to the dorsal surface of each hindpaw in turn and the duration of response (lifting, guarding, licking or biting) was recorded. On a test day, each paw was tested once separated by at least 5 min between tests. The average duration of response from the two hindpaws was then calculated.

2.2.4. Mechanical allodynia

A set of calibrated von Frey hair monofilaments were used to assess the sensitivity to punctate mechanical stimuli. Mice were placed in clear pexiglass chambers on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hindpaw in the ascending order of bending force (range: 0.04–2.0 g). Each hair was applied 5 times per paw, and the number of nocifensive responses (vigourous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The monofilament which produced nocifensive responses greater than 60% of the time was taken as the "threshold".

2.3. Immunocytochemistry

Immunocytochemistry was carried out on EAE tissue taken at three different time points after disease induction. "Onset": lumbar spinal cords taken from mice at clinical grade 1 ($n = 4$); "Peak": lumbar spinal cords taken from mice at clinical grade 3 or 4 ($n = 5$); "Chronic": lumbar spinal cords taken 4 weeks after EAE induction ($n = 3$). Lumbar spinal cords from mice that only received the CFA and pertussis treatments served as controls ($n = 3$). Mice were anesthetized and sacrificed by transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The lumbar enlargement of the spinal cord was removed, post-fixed for 3–4 h and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek® O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20 μm). The following antibodies were used: (i) rabbit anti-CGRP (1:4000, Chemicon, Temecula, CA), (ii) rabbit anti-galanin (1:4000, Chemicon, Temecula, CA), (iii) rat anti-CD3 (1:200, Serotec, Oxford, UK), (iv) rabbit anti-GFAP (1:1000, Dako, Mississauga, ON), (v) rat anti-F4/80 (1:200, Serotec, Oxford, UK). Primary antibodies were visualized using goat anti-rabbit Alexa Fluor®594 secondary antibodies (1:200, Molecular Probes, Eugene, OR).

2.4. Quantification of immunocytochemistry

Images were captured with a Hamamatsu Orca ER camera (Hamamatsu Photonics, Hamamatsu, Japan) using a Leica DMI6000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). All image analysis and cell counting were carried out by an observer blind to the specific experimental conditions of the tissue that was analyzed. The innervation density of CGRP, galanin, GFAP and F4/80 in cross-sections of the lumbar spinal cord from L4 and L5 segments was measured using NIH ImageJ software. Optical density of CGRP, galanin, GFAP and F4/80 immunoreactivity was examined specifically in a standardized region that encompassed the superficial laminae (I–II) of the dorsal horn (three sections per slide, two slides per animal). The level of background staining was determined for each section and subtracted for all optical density measurements. The number of CD3+ positive cells within the grey matter of the superficial dorsal

horn (laminae I–II) was counted in spinal segments from L4 and L5. Left and right dorsal horns were quantified, and an average was calculated per section (three sections per slide, two slides per animal).

2.5. Statistical analysis

Statistical analysis was carried out using Student's *t*-test and two-way repeated measures (RM) ANOVA with Tukey post hoc tests. Significance was set at $P < 0.05$.

3. Results

3.1. Disease progression and neurobehavioural deficits in the MOG_{35-55} model

We first assessed whether there are any observable differences in the disease course of mice with EAE induced with MOG_{35-55} from different sources. Both cohorts of mice showed characteristic clinical deficits that developed between days 13 and 19 post induction. Symptoms began as paralysis in the tail (clinical grade (1) and progressed to more severe clinical deficits corresponding to clinical grade 3 (severe hindlimb weakness with slow righting reflex) or clinical grade 4 (hindlimb paralysis in one hindlimb or both) by day 21. Overall, there was a significant difference in these clinical deficits over the course of the disease between the two groups ($P = 0.02$, two-way RM ANOVA) (Fig. 1A). The mean day of symptom onset was slightly earlier in mice in the EAE "A" cohort (day 15 vs. days 16.5, respectively) and symptom severity was greater in these mice between days 16 and 19 post induction compared to those in the EAE "B" cohort. While clinical deficits tended to diminish in the later stages of the disease in the EAE "B" cohort (mean clinical score of 2.1 between days 24 and 28), they remained severe in mice in the EAE "A" cohort during this phase of the disease (mean clinical score of 2.95 between days 24 and 28) ($P < 0.05$, Tukey post hoc test) (Fig. 1A).

As an adjunct measure of neurological function, we also assessed these mice using the rotorod assay. Mice were trained to walk on the rotorod at a fixed rotational speed (16 rpm) prior to disease induction. All mice tested can carry out this task without any difficulty and can remain on the rotorod for the full duration of the test (180 s). Impairments in gross locomotor ability can therefore be easily detected by a failure to complete this task. Performance on the rototrod test was equally affected in mice from the EAE "A" and EAE "B" cohorts compared to CFA-treated controls (CFA vs. EAE "A" and CFA vs. EAE "B"; $P < 0.001$, two-way RM ANOVA). Deficits in rotorod performance emerged slightly earlier and were more severe in the EAE "A" cohort, but there was no statistical difference between the overall performance of these two groups over time (EAE "A" vs. EAE "B"; $P = 0.38$, two-way RM ANOVA).

Additionally, we analyzed the rotorod performance data between the EAE "A" and EAE "B" cohorts at specific clinical grades over the course of the disease. Overall, rotorod performance was not significantly different between the two cohorts of mice at any specific clinical grades ($P = 0.155$, two-Way RM ANOVA) (Fig. 1C). Deficits in rotorod performance were, however, slightly more pronounced in the EAE "A" cohort at the early stages of disease progression (clinical grades 1 and 2). Rotorod performance became increasingly impaired as these mice progressed to more severe clinical grades 3 and 4 (Fig. 1C). In contrast, in the EAE "B" cohort, rotorod performance was only affected at the more severe clinical grades 3 and 4 compared to their baseline activity (Fig. 1C). These findings demonstrate that subtle differences in neurological impairments can be detected in mice with EAE depending on the source of the antigen (i.e., MOG_{35-55}) used to induce the disease. Therefore, we next asked whether these differences in disease symptoms have any effects on pain behaviours in these mice.

3.2. No change in the sensitivity to noxious heat in the MOG_{35-55} EAE model

We first assessed whether there were any changes in the sensitivity to noxious heat in the hindpaws of mice with EAE. Collectively, mice with EAE (both EAE "A" and EAE "B" groups) showed no significant differences in their withdrawal latencies compared to CFA controls over the majority of the testing period ($P = 0.55$, two-way RM ANOVA) (Fig. 2A). At later stages of the disease (days 19–25), withdrawal latencies began to increase significantly compared to controls ($P < 0.05$, Tukey post hoc test) (Fig. 2A). This hyposensitivity is most likely explained by the significant impairments in gross locomotor ability seen in these mice at this stage of the disease (Fig. 1B and C).

In addition, we analyzed how the specific cohorts of EAE mice responded in this assay at specific stages of disease progression.

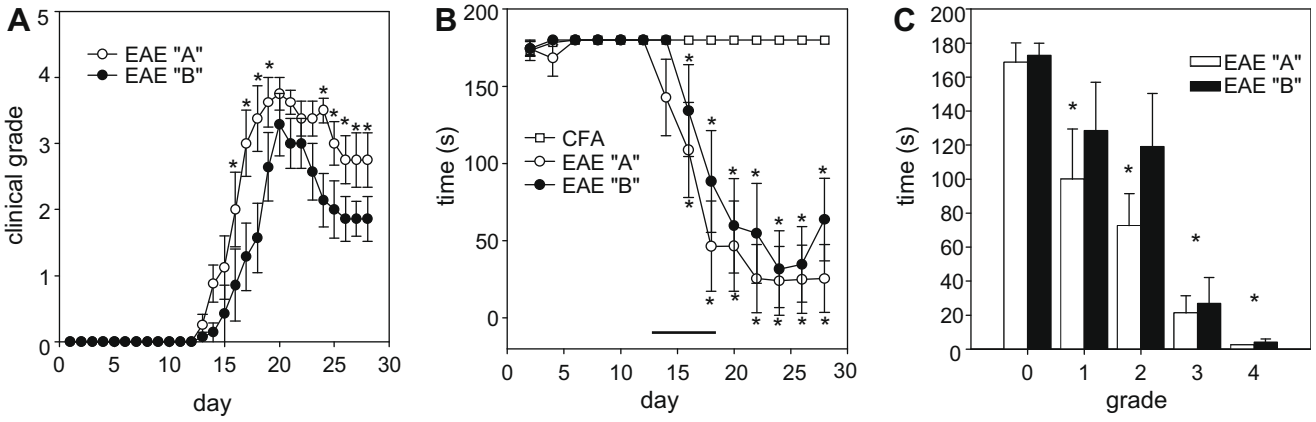

Fig. 1. Disease progression in MOG_{35-55} EAE. (A) Progression of clinical deficits in the EAE "A" and EAE "B" cohorts of mice. Treatments are significantly different ($^*P < 0.05$, two-way RM ANOVA, Tukey post hoc test). (B) Rotorod performance is significantly impaired over time in mice with MOG_{35-55} EAE. Both cohorts are significantly impaired compared to CFA-treated controls ($^*P < 0.05$, two-way RM ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. (C) Rotorod performance at specific clinical grades. Rotorod performance is significantly affected in EAE "A" mice at all stages of disease progression relative to their own baseline performance. Rotorod performance was only affected at clinical grades 3 and 4 in the EAE "B" cohort relative to baseline ($^*P < 0.05$, two-way RM ANOVA, Tukey post hoc test). There were no significant differences in overall rotorod ability between the two cohorts at any clinical grade.

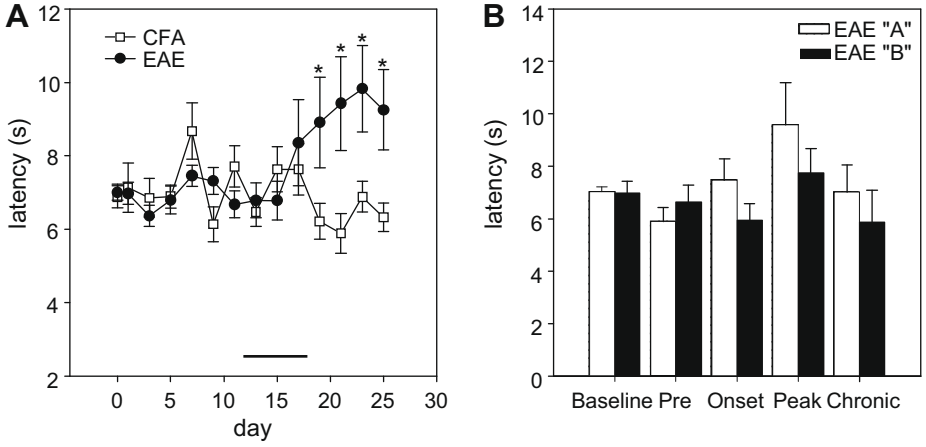

Fig. 2. Sensitivity to noxious heat in MOG_{35-55} EAE. (A) Mean latency to withdraw from a noxious heat source applied to the plantar surface of the hindpaw in MOG_{35-55} EAE mice and CFA-treated controls over time. Overall, there is no significant effect of group on withdrawal latencies over the testing period ($p = 0.55$, two-way RM ANOVA). Withdrawal latencies only become affected in MOG_{35-55} mice in the later stages of the disease ($^{*}P < 0.05$, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. (B) Withdrawal latencies to noxious heat between the two cohorts of EAE mice at specific stages of disease progression. There are no significant differences in responses between the two cohorts.

Responses were assessed at "Baseline" (mean responses prior to disease induction); "Pre-symptomatic" (mean responses from the first day post induction up the first sign of clinical deficit); "Onset" (mean responses from mice at clinical grade 1); "Peak" (mean responses from mice at clinical grades 3 and 4); and in the "Chronic" (the mean responses from mice at clinical grade 2 or lower after having reached clinical grade 3 or 4) phase of the disease. There were no significant differences in the withdrawal latencies to noxious heat between the two cohorts of EAE mice at any stage of disease progression ($P = 0.127$, two-way RM ANOVA) (Fig. 2B). Similarly, withdrawal latencies were not significantly affected at any stage of disease progression within each of the respective cohorts relative to their own baseline responses. These findings suggest that thermal hyperalgesia in the hindpaws is not a major symptom in the MOG_{35-55} EAE model.

3.3. Cold and tactile allodynia are the predominant sensory disturbances in the MOG_{35-55} EAE model

Cold and tactile allodynia are often reported sensory disturbances in patients with MS [36]. We therefore assessed whether similar symptoms can be detected in mice with MOG_{35-55} EAE. Using the acetone test to detect changes in the sensitivity to innocuous cold stimuli, we find a significant difference in the duration of response to acetone application in mice with MOG_{35-55} EAE compared to CFA-treated controls over the testing period ($P = 0.019$, two-way RM ANOVA) (Fig. 3A). Indicative of cold allodynia, responses in EAE mice become significantly prolonged compared to controls early in the course of the disease and persist for several days until severe clinical symptoms appear (Fig 3A). When we assess acetone responses in each EAE cohort separately and at specific stages in the disease, we detect no significant differences in response duration between the two cohorts at any stage of the disease ($P = 0.141$, two-way RM ANOVA). Cold allodynia was equally evident in mice from the EAE "A" and "B" cohorts with significantly prolonged responses in the earliest stages of the disease at the "Pre-symptomatic" and "Onset" phases relative to their respective baseline responses ($P < 0.05$, Tukey post hoc test) (Fig. 3B).

Similarly, MOG_{35-55} EAE mice show a robust tactile allodynia that also becomes apparent prior to any signs of neurological deficit. Withdrawal thresholds to von Frey hair monofilaments drop significantly in mice with EAE compared to CFA-treated controls

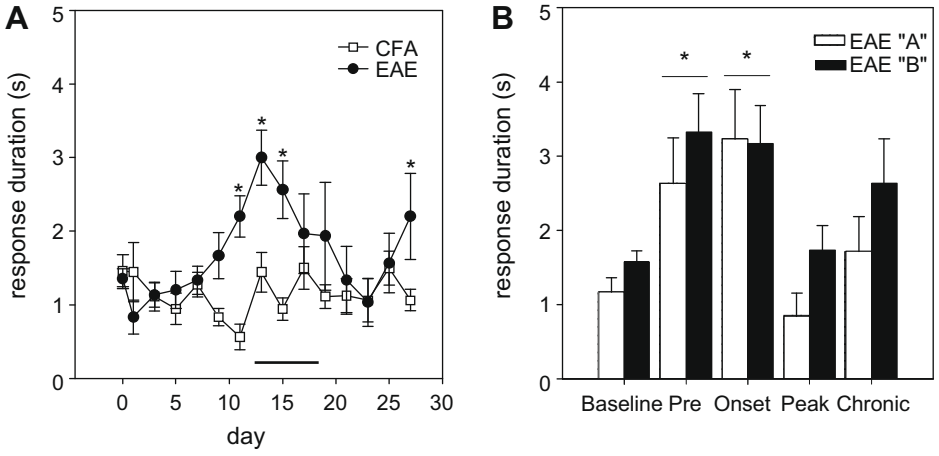

Fig. 3. Sensitivity to innocuous cold in MOG_{35-55} EAE. (A) The duration of response to a single application of acetone to the hindpaw becomes significantly prolonged in MOG_{35-55} EAE mice compared to CFA-treated controls ($^{*}P < 0.05$, two-way RM ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. Note that an increased duration in response is evident just prior to this period. (B) Duration of acetone responses between the two cohorts of EAE mice at specific stages of disease progression. There is no significant difference in the responses between the two cohorts at any stage. The duration of response was increased in both cohorts at the "Pre-symptomatic" and "Onset" stages relative to baseline responses ($^{*}P < 0.05$, two-way RM ANOVA, Tukey post hoc test).

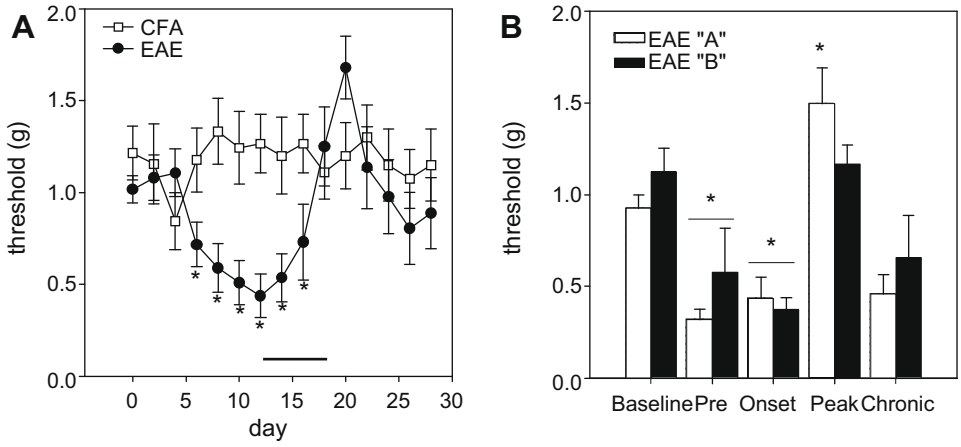

Fig. 4. Sensitivity to punctate mechanical stimuli in MOG_{35-55} EAE. (A) Nociceptive withdrawal thresholds to von Frey hair stimulation are significantly reduced in MOG_{35-55} EAE mice compared to CFA-treated controls ($^{*}P < 0.05$, two-way RM ANOVA, Tukey post hoc test). The black bar indicates the period of symptom "onset" in EAE mice. Note the significant reduction in withdrawal thresholds prior to this period. (B) Withdrawal thresholds between the two cohorts of EAE mice at specific stages of disease progression. There are no significant differences in withdrawal thresholds between the two cohorts at any stage. Withdrawal thresholds are significantly diminished in both cohorts at the "Pre-symptomatic" and "Onset" stages relative to baseline responses. Thresholds in the EAE "A" cohort are elevated relative to baseline at the "Peak" stage of disease ($^{*}P < 0.05$, two-way RM ANOVA, Tukey post hoc test).

beginning by day 5 post induction and persisting until major clinical deficits emerge in most mice by day 15 ($P = 0.004$, two-way RM ANOVA) (Fig. 4A). Like cold allodynia, tactile hypersensitivity was equally evident in both cohorts of mice with EAE. There are no significant differences in withdrawal thresholds between the two cohorts at different stages of the disease ($P = 0.75$, two-way RM ANOVA) (Fig. 4B). Mice in both the EAE "A" and "B" cohorts had similar reductions in their withdrawal thresholds relative to their baseline responses at the "Pre-symptomatic" stage and at disease "Onset" stage ($P < 0.05$, Tukey post hoc test). At the "Peak" stage of disease, allodynia could no longer be detected and withdrawal thresholds were actually elevated compared to baseline responses in both the "A" and "B" groups (Fig. 4B). Again, this is most likely a reflection of the significant impairments in gross locomotor function seen in these mice that hinders proper hindlimb function and makes withdrawal responses difficult to carry out. As clinical symptoms abate in some mice in the "Chronic" phase of the disease, we find that withdrawal thresholds tend to decrease below those seen in the baseline condition (Fig. 4B). Taken together, these findings illustrate that both cold and tactile allodynia are major sensory disturbances that can be detected early in the disease course of the MOG_{35-55} model of EAE. This behavioural hypersen-

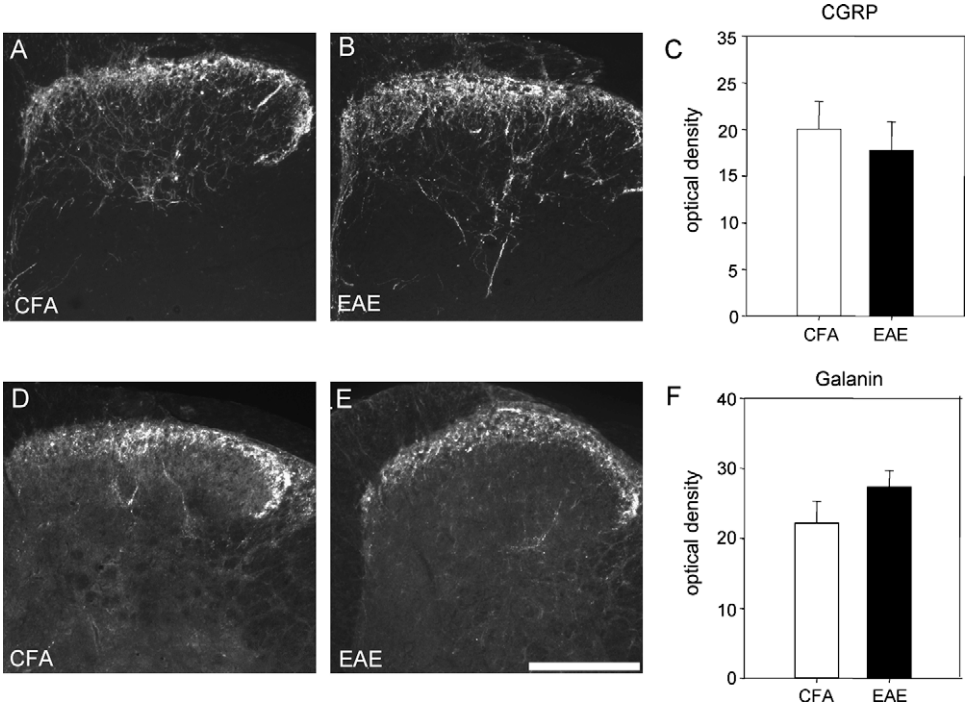

Fig. 5. Expression of sensory neuropeptides in the superficial dorsal horn in MOG_{35-55} EAE. (A and B) Examples of CGRP expression in the superficial dorsal horn from a CFA-treated control (A) and a MOG_{35-55} EAE mouse taken from the chronic phase of the disease (B). (C) There is no significant difference in CGRP expression between the two groups. (D and E) Examples of galanin expression in the superficial dorsal horn from a CFA-treated control (D) and a MOG_{35-55} EAE mouse taken from the chronic phase of the disease (E). (F) There is no significant difference in galanin expression between the two groups. Scale bar in $E = 100$ μm and applies throughout.

sitivity to both innocuous cold and tactile stimuli is independent of the specific degree of neurological deficits that arise in the disease.

3.4. Sensory neuropeptide expression in the spinal dorsal horn in the MOG_{35-55} EAE model

Having established that sensory processing is significantly affected in the MOG_{35-55} EAE model, we next examined potential cellular mechanisms that may underlie these changes. Since there were no significant differences in pain behaviours between the two groups, tissues from EAE "A" and "B" cohorts were pooled for this analysis. We began by examining the expression patterns of two sensory neuropeptides, calcitonin gene-related peptide (CGRP) and galanin, in the superficial dorsal horn from mice with MOG_{35-55} EAE and CFA-treated controls. The expression of these neuropeptides is significantly upregulated in the dorsal horn in conditions associated with neuropathic pain and allodynia [4,19,20,30]. To determine whether similar changes arise in a disease model such as EAE, we used immunocytochemistry and quantified the density of staining for CGRP and galanin in laminae I–II of the superficial dorsal horn of mice with EAE and CFA-treated controls. Neither CGRP nor galanin staining density changed significantly with EAE at any time point during disease progression compared to the expression seen in CFA controls (Fig. 5A–F).

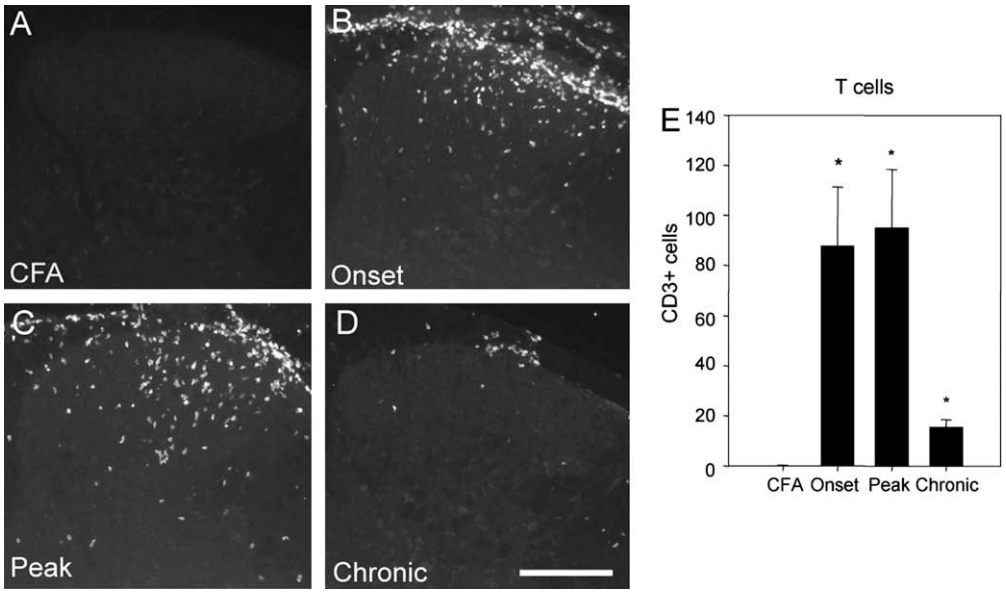

Fig. 6. T cell expression in the superficial dorsal horn in MOG_{35-55} EAE. (A–D) Expression of CD3+ T cells in the superficial dorsal horn from a CFA-treated control (A) and MOG_{35-55} EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. CD3+ cells were never observed in this region in CFA-treated mice. (E) Quantification of CD3+ cells in the superficial dorsal horn. Significant numbers of CD3+ cells are found in this region at all stages of the disease compared to CFA-treated controls ($^{*}P < 0.05$, *t*-test). Scale bar in *D* = 100 μm and applies throughout.

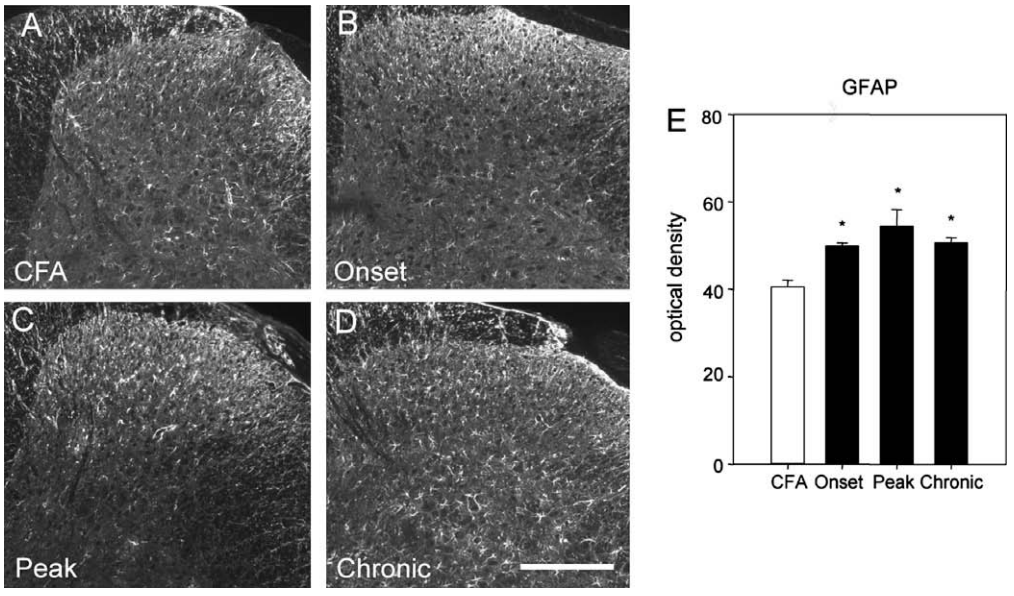

Fig. 7. Astrocyte reactivity in the superficial dorsal horn in MOG_{35-55} EAE. (A–D) GFAP expression in the superficial dorsal horn from a CFA-treated control (A) and MOG_{35-55} EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. (E) There is a slight but significant increase in GFAP expression at all stages of the disease compared to CFA-treated controls ($^{*}P < 0.05$, *t*-test). Scale bar in *D* = 100 μm and applies throughout.

3.5. Inflammation and reactive gliosis in the spinal dorsal horn in the MOG_{35-55} EAE model

Mounting evidence suggests that inflammation and changes in glial cell reactivity in the spinal cord can significantly influence pain sensitivity in a variety of injury models [9,33,41]. While it is well established that the characteristic features of EAE models are inflammation and reactive gliosis in the CNS, little attention has been paid to how these reactions proceed in regions of the spinal cord dedicated to processing sensory inputs. We therefore examined the number of T cells present in the superficial dorsal horn as well as astrocyte and microglia/macrophage reactivity in this region at different stages of EAE disease progression.

Using a CD3 antibody (that detects both CD4+ and CD8+ subsets of T cells), we found significant numbers of T cells in the superficial dorsal horn over the course of the disease (Fig. 6B–D). In contrast, virtually no CD3+ cells were found in the superficial dorsal horn of CFA-treated control mice (Fig. 6A). The mean number of T cells present in the superficial dorsal horn was significantly greater in EAE mice at all disease stages compared to CFA-treated controls (CFA: 0.16 ± 0.1 vs. "Onset": 87.6 ± 23.6; vs. "Peak": 95 ± 23.3; vs. "Chronic": 15.3 ± 3, $P < 0.05$, t-test) (Fig. 6E).

Astrocyte reactivity was also significantly enhanced in mice with EAE compared to CFA controls (Fig. 7A–D). The staining density of GFAP in the superficial dorsal horn was significantly greater in EAE mice at all stages of disease progression but was most evident during the "Peak" phase of the disease ($P < 0.05$, t-test) (Fig. 7E). Similarly, when we analyzed the expression of F4/80 to assess microglia/macrophage reactivity, we found a significant increase in staining density in this region at all stages of disease progression in EAE mice (Fig. 8A–E) ($P < 0.05$, t-test). Unlike T cell and astrocyte responses, however, enhanced microglia/macrophage reactivity was most pronounced in the "Chronic" phase of the disease (Fig. 8D and E). These findings suggest that, similar to other injury models associated with neuropathic pain behaviours, inflammation and reactive gliosis in the spinal dorsal horn may also be a critical mechanism underlying the behavioural hypersensitivity observed in the MOG_{35-55} EAE model.

4. Discussion

It is now recognized that chronic pain is a major symptom associated with MS [2,6,29,31,37]. To date, however, few studies have examined pain-related behaviours in the animal model most commonly used to study the pathophysiology of MS, EAE. We have now characterized the behavioural changes in sensitivity to noxious heat and mechanical stimuli as well as innocuous cold in mice with a chronic-relapsing form of EAE. Using MOG_{35-55} in female C57BL/6 mice, we demonstrate that mice with this form of EAE develop both cold and tactile allodynia early in the disease process, prior to any overt neurological deficits, similar to some patients with MS [31,36]. However, increased sensitivity to noxious heat (thermal hyperalgesia) in the hindpaws does not appear to be a major symptom in this particular EAE model. At the cellular level, we have identified significant changes in glial reactivity and an increased presence of T cells in the superficial dorsal horn as potential underlying mechanisms for the allodynia observed in these mice.

4.1. Using EAE models to study neuropathic pain in MS

Similar to MS, clinical deficits in EAE models can follow either a relapsing–remitting, or a chronic-relapsing, disease course. To date, however, changes in pain sensitivity have only been examined in one type of EAE model [1]. Aicher and colleagues used $PLP_{131-155}$ to induce a relapsing–remitting form of EAE in the SJL/J strain of mice and found that mice with this form of the disease develop a thermal hyperalgesia in the clinically affected body regions (i.e., the tail), which emerges at the peak of neurological deficits and persists for several weeks. In contrast, we found no significant changes in the sensitivity to noxious heat in the hindpaws of mice with MOG_{35-55} EAE. The observed differences in heat sensitivity between the $PLP_{131-155}$ model and the MOG_{35-55} EAE model used here may be accounted for by the different assays used (tail-flick vs. "Hargreaves" plantar test) or they may reflect inherent differences in heat sensitivity between the different mouse strains used in these two models (SJL/J vs. C57BL/6) [28]. However,

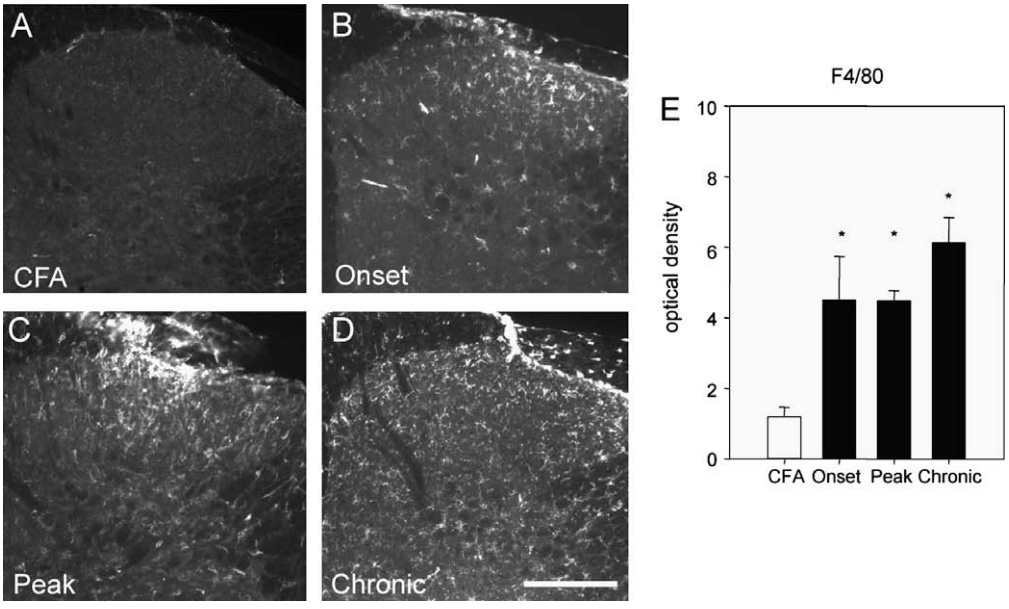

Fig. 8. Microglia/macrophage reactivity in the superficial dorsal horn in MOG_{35-55} EAE. (A–D) F4/80 expression in the superficial dorsal horn from a CFA-treated control (A) and MOG_{35-55} EAE mice at disease "Onset" (B), "Peak" (C) and "Chronic" (D) phases of the disease. (E) There are significant increases in F4/80 expression at all stages of the disease compared to CFA-treated controls (*$P < 0.05$, t-test). Scale bar in D = 100 μm and applies throughout.

a recent clinical report examining pain in MS has highlighted a strong association between the type and severity of pain experienced with MS subtype [11]. In addition, recent data from animal studies using a viral model of MS have shown that SJL/J mice infected with Theiler's murine encephalomyelitis virus (TMEV) exhibit distinct changes in pain sensitivity to both thermal and mechanical stimuli that are influenced by gender [23]. Therefore, it cannot be ruled out that different animal models used to study MS (i.e., $PLP_{131-155}$ EAE vs. MOG_{35-55} EAE; EAE vs. TMEV) may generate their own distinct pain "phenotype".

Interestingly, we observed differences in disease course and symptom severity in different cohorts of animals immunized with MOG_{35-55}. Even with these differences in the clinical manifestations of the disease, pain behaviours were affected equally in the two cohorts of mice. This suggests that, in the MOG_{35-55} EAE model, changes in sensory function and the development of neuropathic pain behaviours are independent of symptom severity and the clinical course of the disease.

Allodynia to cold and tactile stimuli was also evident in mice with MOG_{35-55} EAE. The most striking observation from these experiments was the development of these symptoms prior to any signs of overt neurological dysfunction. It has been noted that in a large proportion of MS patients with neuropathic pain, that pain can be a major symptom just prior to or immediately at the onset of clinical deficits in the disease [31]. These similarities highlight the usefulness of using the MOG_{35-55} EAE model to study neuropathic pain in MS.

4.2. Potential cellular mechanisms for neuropathic pain in EAE-sensory neuropeptides

To assess the underlying mechanisms that mediate the development of cold and tactile allodynia in the MOG_{35-55} EAE model, we examined the innervation patterns of two sensory neuropeptides commonly associated with pain processing, CGRP and galanin, in the spinal dorsal horn. Enhanced expression or sprouting of $CGRP^+$ axons in the dorsal horn has been identified as an important anatomic substrate for allodynia and autonomic dysreflexia in spinal cord injury models [4,19,30]. Similarly, neuropathic pain in peripheral nerve injury models is associated with significant alteration in the expression of galanin in the superficial dorsal horn [20]. However, in mice with MOG_{35-55} EAE we found no changes in CGRP or galanin expression in this region. Therefore, alterations in sensory processing in the MOG_{35-55} EAE model cannot be accounted for by differences in the expression patterns of these "classical" sensory neuropeptides at the spinal level.

4.3. Potential cellular mechanisms for neuropathic pain in EAE inflammation and reactive gliosis

Although we found no changes in sensory neuropeptide expression in the superficial dorsal horn, we did observe significant numbers of T cells and enhanced reactivity of astrocytes and microglia/macrophages in this region at every stage of the disease ("Onset", "Peak" and "Chronic" phases). Non-neuronal cells such as microglia/macrophages, astrocytes and T cells are now recognized to be integral for the generation and maintenance of central sensitization and neuropathic pain [26,33,41].

T cells are known to traffic into the superficial dorsal horn after peripheral nerve injury [38] and mice lacking mature T cells (athymic "nude" mice) do not develop neuropathic pain after peripheral nerve injury [27]. Endoneurial T cells have also been implicated as a potential underlying mechanism of neuritic pain [10]. In MOG_{35-55} EAE mice, CD3+ T cells are abundant in the superficial dorsal horn early in the disease correlating with the development of allodynia. T cells most likely affect sensory processing through their release of pro-inflammatory cytokines that can directly sensitize dorsal horn neurons [16]. There is also increasing evidence that T cells can produce and secrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [18,34]. While this suggests that some infiltrating leukocytes in MS may have a neuroprotective role, BDNF is also known to modulate synaptic transmission and neuronal excitability [14,21] and is a key mediator in the genesis of central sensitization and pain hypersensitivity [8,17,22,24]. Whether specific subsets of T cells (i.e., CD4+ vs. CD8+; Th1 vs. Th2) differentially affect pain processing in EAE models is an important question that remains to be addressed.

Reactive gliosis in the superficial dorsal horn was also a prominent feature of mice with MOG_{35-55} EAE. Increased reactivity of astrocytes and microglia/macrophages was evident at disease onset and, particularly for microglia, remained significantly elevated into the chronic stages of the disease. Much like T cells, reactive glial cells can modulate spinal excitability and mediate pain hypersensitivity through their production and release of cytokines and growth factors such as BDNF [8,9]. Similar to the neuropathic pain that arises as a result of peripheral nerve lesions, sensitization of spinal pain pathways and the consequent allodynia observed in the MOG_{35-55} EAE model may be triggered by increased activity in these non-neuronal cells in the superficial dorsal horn [7,12]. In the case of peripheral nerve injury, it has been suggested that injury in the periphery triggers the neuronal production of mediators such as ATP, the chemokines CCL2 (MCP-1) or fractalkine that directly activate spinal microglia [25,39,40,42]. Whether similar mechanisms are engaged in EAE models needs to be determined. Given that there is little evidence for direct injury to the peripheral nervous system in the MOG_{35-55} EAE model, the most likely source of spinal glial activation in the disease is from the initial trafficking of T cells into the CNS. These early inflammatory events may then set off the cascade of responses from astrocytes and microglia that leads the observed behavioural hypersensitivities.

5. Conclusions

Taken together, our results demonstrate that the MOG_{35-55} EAE model is a useful tool to study neuropathic pain in MS. Future studies will now be aimed at addressing the specific signaling pathways and cellular mechanisms that mediate the changes in sensory function observed in the disease. A better understanding of these mechanisms will help to tailor future therapies to treat the often neglected symptoms of neuropathic pain in MS.

Acknowledgments

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A diminished response to formalin stimulation reveals a role for the glutamate transporters in the altered pain sensitivity of mice with experimental autoimmune encephalomyelitis (EAE)

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) in which neuropathic pain is now recognized as a major symptom. To date, few studies have examined the underlying mechanisms of neuropathic pain in MS. Recently we showed that in a chronic-relapsing animal model of MS, experimental autoimmune encephalomyelitis (EAE), characteristic neuropathic behaviours develop. However, responses to persistent noxious stimuli in EAE remain unexplored. We, therefore set out to characterize the changes in pain sensitivity in our EAE model to subcutaneous injection of formalin. We show here that female C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG_{35-55}) display a significant decrease in elicited pain behaviours in response to formalin injection. These effects were found to involve dysregulation of the glutamatergic system in EAE. We show here that these effects are mediated by decreased glutamate transporter expression associated with EAE. Our findings demonstrate that dysregulation of glutamate transporter function in EAE mice is an important mechanism underlying the abnormal pain sensitivity in response to persistent noxious stimulation of mice with EAE and also sheds light on a potential mechanism underlying neuropathic pain behaviours in this model.

1. Introduction

Chronic pain is now recognized as a major symptom associated with the autoimmune demyelinating disease Multiple Sclerosis (MS) [2,3,35]. Common pain syndromes in MS patients include trigeminal neuralgia, musculoskeletal and neuropathic pain [19]. Of these pain syndromes, neuropathic pain is the least understood and most difficult to treat [34]. Neuropathic pain states are thought to arise from the process of central sensitization in the spinal cord [41]. Central sensitization is triggered by inputs from nociceptive afferents and is associated with a reduced threshold for activation of dorsal horn neurons to noxious stimulation [27].

The activation of glutamate receptors has been strongly implicated in the generation of central sensitization [5]. Glutamate receptor activation can be regulated by a family of Na^+ dependent high affinity glutamate transporters [7,10,11]. In the spinal cord, three isoforms of glutamate transporters have been described (EAAT-1, EAAT-2 and EAAT-3). EAAT-1 and EAAT-2 are present in glial cells at perisynaptic sites and carry out the majority of glutamate uptake activity [7]. In the spinal cord, these transporters are concentrated in the superficial dorsal horn [36] an area important for sensory input and pain processing. Dysregulation of glutamate transporter activity is associated with a persistent elevation in ambient glutamate and has been implicated as a potential mechanism for central sensitization and neuropathic pain following peripheral nerve injury [33].

Recently, neuropathic pain behaviours have been described and characterized in two commonly used animal models to study MS, experimental autoimmune encephalomyelitis (EAE) and the Theiler's murine encephalomyelitis virus (TMEV) model [1,15,24]. Mice with EAE and TMEV display characteristic neuropathic pain behaviours such as hyperalgesia to noxious heat and allodynia to cold and mechanical stimuli [1,15,23]. However, the underlying mechanisms that generate neuropathic pain in these models remain unknown. In addition, it remains unclear how the responses to more persistent noxious stimuli are affected in these animals. Given that mice with EAE exhibit a robust allodynia to both cold

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and mechanical stimulation [24], we predicted that they would show a similar hypersensitivity in response to more intense noxious stimulation. To our surprise, mice with EAE showed a significant decrease in elicited pain behaviours in response to subcutaneous injection of formalin, a behavioural model of injury-induced central sensitization [6]. We show here that these effects are mediated by decreased glutamate transporter expression associated with the disease. Our findings demonstrate that dysregulation of glutamate transporter function is an important mechanism underlying the abnormal pain sensitivity of mice with EAE.

2. Methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with the approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta.

2.1. Induction of EAE

A total of 54, 10–12 weeks old female C57BL/6 mice were used. Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. We induced EAE in mice with myelin oligodendrocyte glycoprotein 35–55 (MEVGWYRSPFSRVVHLYRNGK; MOG_{35-55}) obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB). EAE was induced by subcutaneous immunization with 50 μg of MOG_{35-55} emulsified in complete Freund's adjuvant (CFA), (Sigma–Aldrich, Oakville, ON). The CFA was supplemented with an additional 4-mg/ml heat killed *Mycobacterium* tuberculosis H37Ra (Difco Laboratories) to a final concentration of 5 mg/mL. An intraperitoneal injection of 300 ng Pertussis toxin (Sigma–Aldrich, Oakville, ON) was administered at the time of induction and again 48 h later. Control mice were treated with CFA (as above) and Pertussis toxin alone ($n = 28$).

2.2. EAE assessment

Mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1, flaccid or paralyzed tail. All experimental procedures were carried out once mice reached a score of clinical Grade 1. We have shown previously that at this stage of the disease, mice with EAE display signs of both cold and tactile allodynia but gross locomotor behaviour is unaffected [24]. To confirm that mice with EAE had no gross locomotor deficits that would interfere with the behavioural responses to subcutaneous formalin, they were tested on a rotorod at a fixed rotational speed of 16 rpm. Mice were placed on the rotorod and the latency to fall off was determined. The maximum time for a trial was set at 180 s. Mice were trained on the rotorod for two consecutive days prior to disease induction to become familiar with the task. Impairments in gross locomotor function are revealed when animals can no longer remain on the rotorod for the duration of the trial.

2.3. Formalin test

Prior to testing, each animal was allowed to acclimatize to the testing apparatus. The apparatus was an observation box (25 cm × 23 cm × 15 cm) made of clear plexi-glass with a raised platform to allow for an unobstructed view of the hind paw. After acclimatization, mice were lightly restrained and given a 30 μl subcutaneous injection of a 0.5% formaldehyde in saline solution into the plantar surface of one hind paw. Each mouse was then placed back into the observation box and nociceptive behaviours were monitored and timed in 5-min blocks for a 30-min period. Nociceptive behaviours were defined as licking or lifting of the injected paw, flinching and vigorous shaking. The total time (in seconds) mice spent exhibiting nociceptive behaviours was determined for the first phase (0–15 min) and second phase (15–30 min) of the response (CFA $n = 14$; EAE $n = 9$).

2.4. Drug treatments

Cohorts of mice were treated with the drug (*R*)-(−)-1-methyl-1-nicotinoyl-2-pyrazoline (MS-153) [38], a compound known to promote glutamate transporter activity [20,31]. Mice were treated with 10 mg/kg I.P. 20 min prior to formalin testing (see above) (CFA $n = 5$; EAE $n = 6$). A separate cohort of mice (CFA $n = 3$; EAE $n = 3$) were treated with MS-153 (10 mg/kg I.P.) and euthanized 1 h later without any stimulation to assess FOS expression (see Section 2.5 below). In a separate experiment, mice (CFA $n = 5$; EAE $n = 6$) were treated with LY-341495 (0.25 mg/kg I.P.) (Tocris) a selective group II metabotropic glutamate receptor (mGluR) antagonist [13] 20 min prior to formalin testing.

2.5. Immunocytochemistry

Immunocytochemistry was carried out on CFA or EAE mice at Clinical Grade 1. Spinal cords were taken 1 h after MS-153 treatment without any further stimulation or 1 h after formalin injection. Mice were euthanized with high dose pentobarbital (Euthanyl: 240 mg/ml) and transcardiac perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The lumbar enlargement of the spinal cord was removed, post-fixed for 3–4 h and then transferred to a 30% sucrose solution in 0.1 M PB. Spinal cords were embedded in Tissue Tek® O.C.T (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB), frozen on liquid nitrogen and processed for cryostat sectioning (20 μm). The primary antibody used was goat anti-rabbit *c-fos* (1:1000, Cell Signaling, Danvers, MA). The primary antibody was visualized using goat anti-rabbit Alexa Fluor®594 secondary antibodies (1:200, Molecular Probes, Eugene, OR).

2.6. Quantification of immunocytochemistry

Images were captured with a Zeiss Axiocam MRm camera (Carl Zeiss, Oberkochen, Germany) using a Zeiss Observer.Z1 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All image analysis and cell counting were carried out by an observer blind to the specific experimental conditions of the tissue being analyzed. FOS positive cells were identified in the superficial dorsal horn (lamiae I–III). Cells were counted from dorsal horns ipsilateral and contralateral to the injection. For FOS quantification from unstimulated mice, FOS positive cells were counted from both dorsal horns, pooled and then averaged. Three sections per slide, 2 slides per animal were analyzed.

2.7. Western blots

Protein samples (20 μg) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked in 5% milk in PBS–Tween 20 (PBS-T) (0.05%), and then incubated overnight at 4 °C in primary antibodies EAAT-1 and EAAT-2 (1:500, Santa Cruz Biotechnology), $pNR1^{Ser896}$, $pNR1^{Ser897}$ (1:1000, Cedarlane Laboratories) and NR1 (1:1000, Sigma), diluted in PBS-T. The membranes were then washed in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit; Jackson ImmunoResearch) diluted at 1:50,000. The membranes were washed and binding of

HRP-conjugated secondary antibodies detected using chemiluminescence. (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer.) Membranes were re-probed with monoclonal mouse anti-β-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

2.8. Statistical analysis

Statistical analysis was carried out using the Student's *t*-test and one-way ANOVA with Tukey post hoc tests. Significance was set at $P < 0.05$.

3. Results

3.1. Diminished pain behaviours in response to formalin stimulation in EAE mice

To determine how mice with EAE respond to a persistent noxious stimulus we assessed their behaviour in the formalin test [4]. Prior to testing, mice with EAE (clinical Grade 1) were assessed for gross locomotor function using the rotorod test. No significant deficits in gross locomotor function or coordination were observed in these animals at this stage of the disease (Fig. 1A, also see [24]). Following formalin injection, mice with EAE showed significantly reduced pain behaviours when compared to the CFA control mice. Decreased nociceptive behaviours were evident in both Phase 1 ($P = 0.001$, *t*-test) (Fig. 1B) and even more strikingly in Phase 2 of the formalin response ($P < 0.001$, *t*-test) (Fig. 1C).

3.2. Diminished glutamate transporter expression in EAE mice

Second phase nociceptive behaviours in the formalin test are related to activity at glutamate receptors [43]. We therefore examined the expression of key regulators of glutamate receptor activity, the glutamate transporters [7] in mice with EAE. We began by examining glutamate transporter expression in the spinal cord of EAE mice in the absence of any noxious stimulation. Western blots determined that in the spinal cords of mice with EAE at clinical Grade 1, there is a significant reduction of EAAT-1 ($P = 0.001$, *t*-test) (Fig. 2A and B). There is an even more dramatic reduction in EAAT-2 expression, which is decreased to approximately 50% of the levels seen in CFA control mice ($P < 0.001$, *t*-test) (Fig. 2C and D).

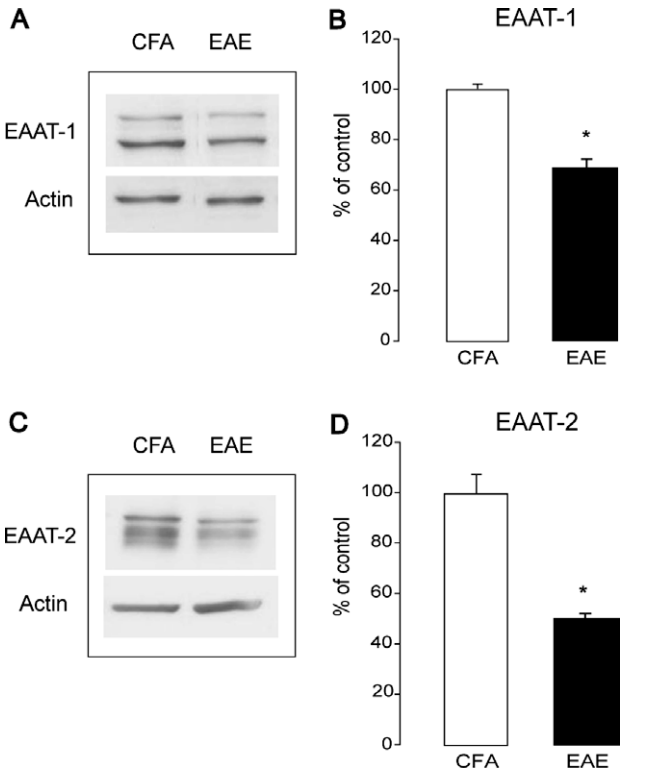

Fig. 2. Spinal EAAT-1 and EAAT-2 expression in MOG_{35-55} EAE mice. (A) There is significantly diminished expression of EAAT-1 in the spinal cord of EAE mice at disease onset compared to CFA controls mice. (B) Quantification of the Western blots for EAAT-1 (*$P = 0.001$, *t*-test). (C) EAAT-2 glutamate transporter expression is also significantly diminished in the spinal cord of EAE mice at disease onset when compared to CFA controls. (D) Quantification of the Western blots for EAAT-2 (*$P < 0.001$, *t*-test).

3.3. Enhanced phosphorylation of NMDA receptor subunits in spinal cords of EAE mice

Given the down regulation of glutamate transporters in EAE mice we next wanted to assess how this might affect activity at glutamate receptors. Phosphorylation of the NR1 subunit of the NMDA receptor is known to correlate with changes in nociceptive behaviours [44]. In the absence of any noxious stimulation, phosphorylation of the NR1 subunit at both the serine 896 and serine

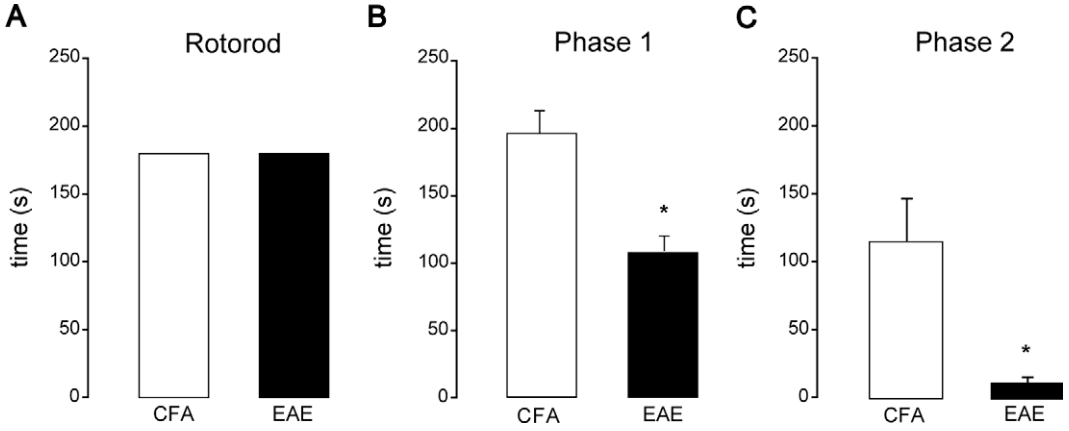

Fig. 1. Responses to formalin injection in MOG_{35-55} EAE mice. (A) Gross locomotor behaviour is not affected in EAE mice at clinical Grade 1. Mice with EAE show no impairments in the rotorod test at this stage of the disease. (B) Duration of nociceptive responses for Phase 1 (0–15 min after formalin injection). The response to formalin is significantly decreased in EAE mice compared to CFA-treated controls (*$P = 0.001$, *t*-test). (C) Duration of formalin responses in Phase 2 (15–30 min after formalin injection). Responses are significantly decreased in EAE mice compared to CFA control mice (*$P < 0.001$, *t*-test).

897 sites is significantly greater in the spinal cord of mice with EAE compared to CFA controls. NR1 phosphorylation at serine 896 was found to be 72% higher in EAE spinal cords compared to CFA control mice ($P < 0.001$, t-test) (Fig. 3A and D) while phosphorylation at the serine 897 site was elevated by 65% ($P < 0.001$, t-test) (Fig. 3B and E). The levels of total NR1 protein were slightly reduced in the spinal cord of EAE mice but these differences were not significant (Fig. 3C and F).

3.4. Increased cellular activation in the spinal cords of EAE mice

To determine what might account for the increased phosphorylation of NMDA receptors in the un-stimulated EAE spinal cord, we assessed the patterns of ongoing cellular activity in the superficial dorsal horn of mice with EAE and CFA controls. Using the protein product of the immediate early gene *c-fos* as a marker for cellular activity [12], we find that in the absences of any noxious stimulation, FOS expression is significantly higher throughout the dorsal horn of mice with EAE compared to CFA controls (mean EAE FOS+ cells: 112 ± 9 vs. CFA FOS+ cells: 20 ± 6, $P < 0.001$, one-way ANOVA, Tukey post hoc test) (Fig. 4A, B and E).

To establish if a loss of glutamate uptake capacity might account for this increase in cellular activation in the dorsal horn of EAE mice we assessed FOS expression after treating mice with MS-153, a compound that promotes glutamate transporter activity [20,31]. In EAE mice, MS-153 treatment leads to a significant reduction in FOS positive cells in the dorsal horn compared to the untreated EAE spinal cord (EAE FOS+ cells: 112 ± 9 vs. EAE/MS-153 FOS+ cells: 77 ± 8, $P = 0.04$, one-way ANOVA, Tukey post hoc test) (Fig. 4C–E). FOS expression does however, remain significantly higher in these mice compared to CFA controls (EAE + MS-153 vs. CFA, $P = 0.003$; EAE + MS-153 vs. CFA + MS-153, $P = 0.01$, one-way ANOVA, Tukey post hoc test) (Fig. 4E) suggesting that additional factors also contribute to the enhanced basal activation of cells in the dorsal horn of EAE mice.

3.5. Cellular activation in response to formalin stimulation

We next assessed how cells in the dorsal horn of EAE mice respond to intense noxious stimulation with formalin. As expected, CFA control mice show a dramatic (approximately fourfold) increase in the number FOS positive cells in the superficial dorsal horn ipsilateral to formalin stimulation (Fig. 5A, compare to Fig. 4A). In contrast, formalin stimulation triggers little change in the activation of cells in the dorsal horn of EAE mice. There is virtually no change in FOS expression compared to baseline levels (Fig. 5B, compare to Fig. 4B). Thus, in agreement with their behavioural phenotypes, CFA control mice are significantly more responsive at the cellular level after formalin stimulation than mice with EAE ($P = 0.001$, t-test) (Fig. 5C).

3.6. Decreased phosphorylation of NMDA receptor subunits in the spinal cord of EAE mice in response to formalin stimulation

In the absence of stimulation, EAE mice display enhanced cellular activation and phosphorylation of NR1 subunits. Following formalin stimulation they become hypo-responsive behaviourally and have little change in their patterns of cellular activation. Therefore, we next assessed how phosphorylation of NMDA receptor subunits is affected by formalin stimulation in EAE mice. Following formalin injection there is a significant decrease in the phosphorylation at the serine 896 site of the NR1 subunit in the spinal cords of EAE mice compared to CFA controls ($P = 0.035$, t-test) (Fig. 6A and C). Phosphorylation at the serine 897 site of the NR1 subunit remains elevated in the EAE spinal cord after formalin stimulation but is no longer significantly different from the levels seen in CFA control mice ($P = 0.607$, t-test) (Fig. 6B and D). Taken together, these results

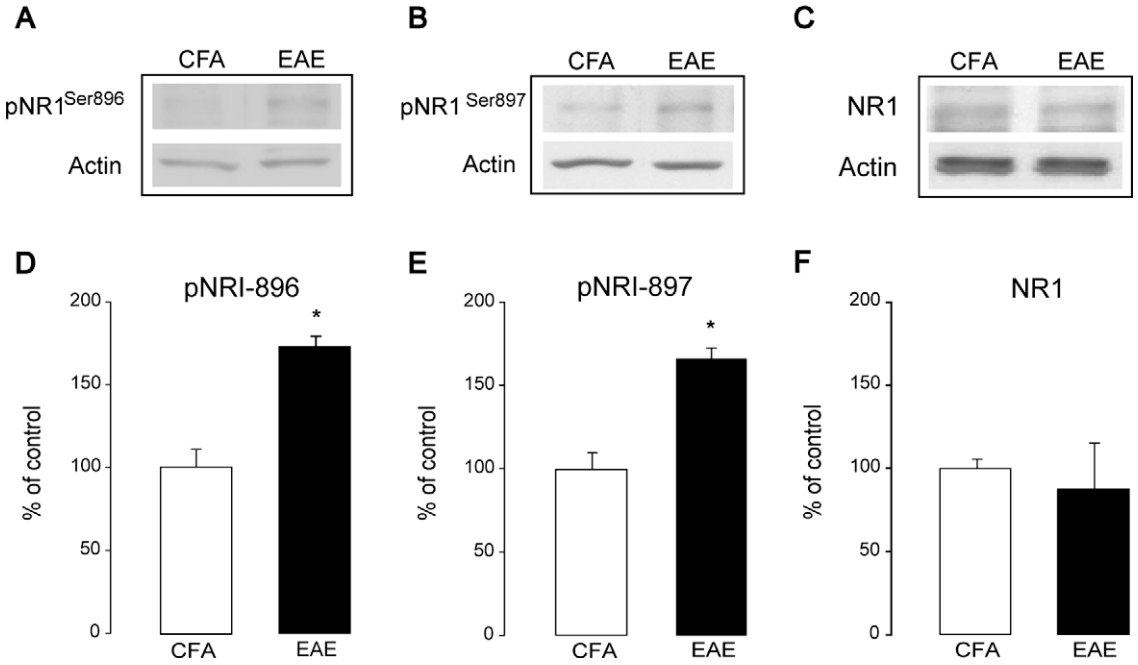

Fig. 3. Phosphorylation of NR1 subunits in the spinal cord of MOG_{35-55} EAE mice. (A) There is a significant increase in the phosphorylation of NR1 at Ser896 in EAE mice at disease onset when compared to CFA controls. (B) Phosphorylation of NR1 at Ser897 is significantly increased in EAE mice at disease onset when compared to CFA controls. (C) Total NR1 expression is relatively unaffected in EAE spinal cords compared CFA controls. (D) Quantification of the Western blots for $NR1^{Ser896}$ phosphorylation (*$P < 0.001$, t-test). (E) Quantification of the Western blots for $NR1^{Ser897}$ phosphorylation (*$P < 0.001$, t-test). (F) Quantification of the Western blots for NR1. There is no significant different between the groups ($P > 0.05$, t-test).

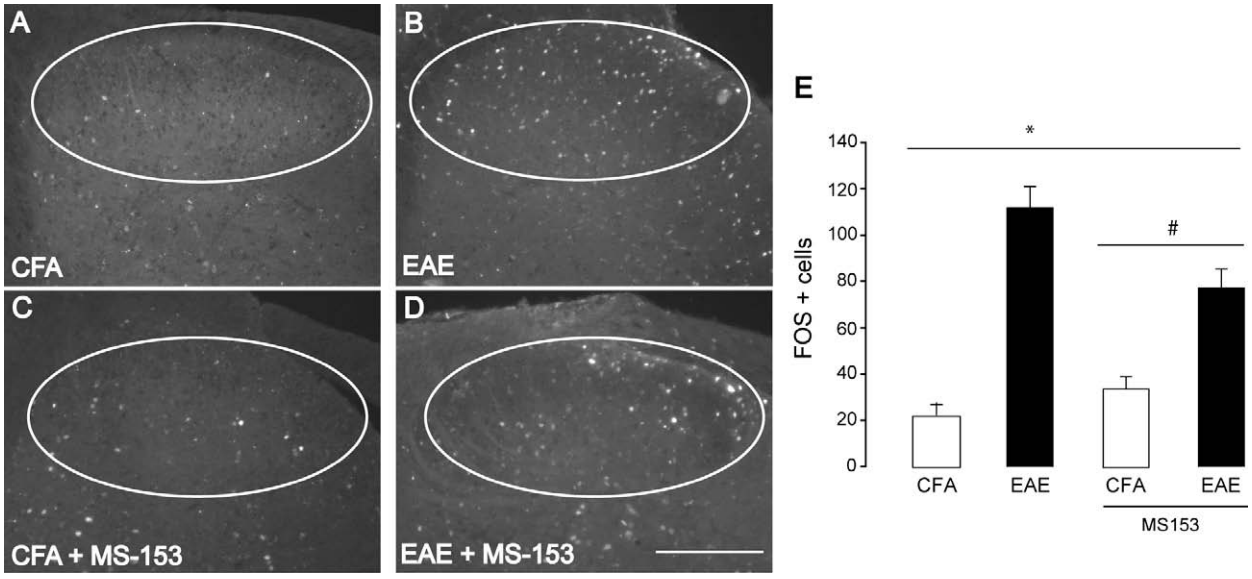

Fig. 4. FOS expression in the spinal dorsal horn of MOG_{35-55} EAE mice. (A and B) Representative images of FOS expression in the superficial dorsal horn from CFA (A) and EAE (B) mice in the absence of stimulation. (C and D) Representative images of FOS expression in the superficial dorsal horn from CFA (C) and EAE (D) mice after treatment with MS-153 treatment in the absence of stimulation. (E) Quantification of FOS positive cells in the superficial dorsal horn. There is a significant increase in FOS expression in the dorsal horn of EAE mice compared to CFA controls. FOS expression is significantly decreased in EAE mice treated with MS-153 when compared to EAE mice without MS-153 treatment. FOS expression is still significantly higher in EAE + MS-153 mice compared CFA controls ($^{*}P = 0.01$, one-way ANOVA, Tukey post hoc test, $^{\#}P = 0.01$, one-way ANOVA, Tukey post hoc test). Scale bar in *D* = 200 μm and applies throughout.

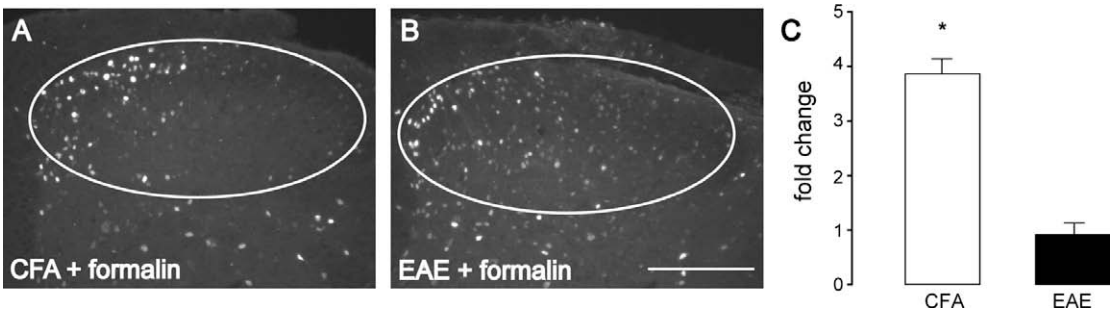

Fig. 5. FOS expression in dorsal horn of MOG_{35-55} EAE mice after formalin. (A and B) Representative images of FOS expression in the ipsilateral superficial dorsal horn from CFA (A) and EAE (B) mice after formalin injection. (C) Relative to the un-stimulated condition there is a fourfold increase in FOS expression in CFA mice after formalin injection. Relative to the un-stimulated condition, FOS expression remains unchanged in the EAE spinal cord after formalin injection ($^{*}P = 0.001$, *t*-test).

suggest that in response to intense noxious stimulation, inhibitory signaling pathways may be engaged more readily in EAE mice.

3.7. Normalization of formalin responses in EAE mice

Decreased glutamate transporter expression can be associated with significant increases in ambient glutamate levels in the spinal cord [14,33]. Given the reduced levels of glutamate transporters in EAE mice, we hypothesized that following formalin stimulation, levels of extracellular glutamate might be elevated sufficiently so that pre-synaptic inhibitory mGluRs become activated more readily [13,42]. Increased activation of these inhibitory pathways in EAE mice could then account for the lack of responsiveness to formalin stimulation. To investigate this, we treated mice with an mGluR2/3 antagonist, LY-341495 prior to formalin injection. The nociceptive responses to formalin injection in EAE mice receiving LY-341495 were indistinguishable from CFA-treated controls. Nociceptive behaviours were normalized in EAE mice compared to CFA controls in both phases of the formalin response (Phase 1: $P = 0.183$, *t*-test) (Phase 2: $P = 0.387$, *t*-test) (Fig. 7A and B).

To verify that these effects are mediated by a dysregulation of glutamate transporters in EAE, we assessed formalin responses after treatment with the drug MS-153. Like LY-341495, treatment with MS-153 was found to normalize the behavioural response to formalin to that of CFA controls (Phase 1: $P = 0.156$, *t*-test) (Phase 2: $P = 0.668$, *t*-test) (Fig. 7C and D). These results demonstrate that the diminished responsiveness to formalin stimulation in EAE can be accounted for by a disturbance in the glutamate uptake system.

4. Discussion

Previously, we have shown that mice with EAE exhibit a robust allodynia in response to acute application of innocuous cold and mechanical stimuli [24]. We have now examined how these mice respond to a more persistent and intense noxious stimulus using the formalin model of injury-induced sensitization [4]. Unexpectedly, mice with EAE were hypo-responsive in this model, exhibiting significantly less nociceptive behaviours during both phases of the response. We show that the lack of behavioural response to formalin stimulation in these mice can be accounted for by a de-

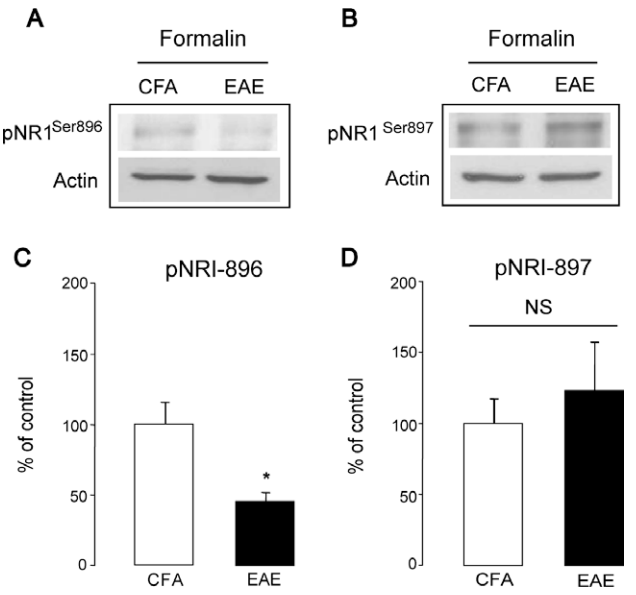

Fig. 6. NR1 subunit phosphorylation after formalin injection. (A) There is a significant decrease in NR1^{Ser896} phosphorylation after formalin injection in EAE mice when compared to spinal cords from CFA controls. (B) Phosphorylation of NR1^{Ser897} is not significantly different between CFA and EAE spinal cords after formalin injection. (C) Quantification of the Western blots for NR1^{Ser896} phosphorylation (*P = 0.035, t-test). (D) Quantification of the Western blots for NR1^{Ser897} phosphorylation (P = 0.607). NS = not significant.

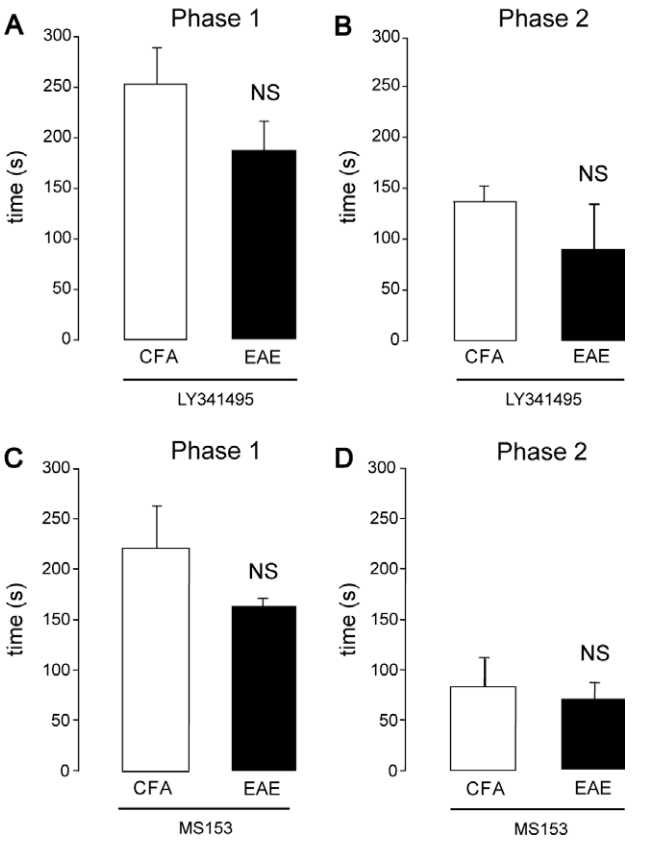

Fig. 7. Responses to formalin injection in EAE mice after treatment with LY-341495 or MS-153. (A) Duration of nociceptive responses in Phase 1 of the formalin test after LY-341495 treatment. There is no significant difference in displayed pain behaviours between the CFA control and EAE mice (P = 0.183). (B) Duration of nociceptive responses in Phase 2 of the formalin test after LY-341495 treatment. There is no significant difference in displayed pain behaviours between the two groups (P = 0.387). (C) Duration of nociceptive responses in Phase 1 of the formalin test with MS-153 treatment. There is no significant difference in nociceptive responses between CFA and EAE mice (P = 0.156). (D) Duration of nociceptive responses in Phase 2 of the formalin test with MS-153 treatment. There is no significant difference in nociceptive responses between CFA and EAE mice (P = 0.668). NS = not significant.

creased expression of the glutamate transporters, EAAT-1 and EAAT-2 in the spinal cord. Deficits in glutamate uptake arising from the downregulation of EAAT-1 and EAAT-2 in EAE mice was predicted to allow excessive amounts of extracellular glutamate to accumulate and engage pre-synaptic inhibitory mGluRs more readily after formalin stimulation. By treating EAE mice with LY-341495, an antagonist for inhibitory group II mGluRs, or by promoting glutamate transporter activity with the drug MS-153, we were able to normalize the formalin response in EAE mice. Importantly, we also show that in the absence of noxious stimulation, the spinal cords of EAE mice display signs of ongoing cellular activation and hyperexcitability that is mediated in part by the decreased levels of EAAT-1 and EAAT-2. These findings have direct implications for the underlying causes of neuropathic pain in MS.

The glutamate transporters are important for regulating the levels of extracellular glutamate at the synapse. Decreased expression of the glutamate transporters has been observed in post mortem samples from patients with MS and rats with EAE [22,25,39]. Glutamate transporters have also been found to be decreased in other pathologies that are associated with neuropathic pain such as after spinal cord and peripheral nerve injury [36]. Spinal cord injury, MS and peripheral nerve injury are all pathologies associated with an increased inflammatory response in the CNS. Inflammation and reactive gliosis are recognized as integral processes in triggering central sensitization and neuropathic pain [18,30,40]. Inflammatory cytokines are also known to influence glutamate transporter expression *in vitro* [37]. Previously, we have demonstrated that mice with EAE show significant increases in the reactivity of microglia/macrophages along with high numbers of T-cells in the dorsal horn of the spinal cord even at the earliest stages of disease [24]. Given that these cell types are a major source for many of the cytokines known to influence transporter expression and trafficking, it is also likely that this early inflammatory response in the dorsal horn can account for the downregulation of EAAT-1 and EAAT-2 observed here.

A physiological consequence of decreased glutamate transporter expression in EAE mice would be an elevation in ambient extracellular glutamate levels at the synapse. Loss of these transporters could also lead to "spillover" of glutamate into nearby or adjacent synapses and the activation of distant cells. Using FOS as a marker of cellular activation, we find that, even in the absence of noxious stimulation, mice with EAE have a high level of ongoing cellular activity that is distributed throughout the dorsal horn of the spinal cord. This pattern of activity could be significantly reduced following treatment with MS-153, a compound that promotes glutamate uptake activity [20,31]. While MS-153 could reduce the levels of FOS expression compared to untreated EAE mice, FOS expression was still significantly elevated in EAE mice treated with MS-153 compared to CFA controls. This suggests that additional factors also contribute to the ongoing cellular activation in the dorsal horn of EAE mice. The most likely source for these signals is the high level of reactive gliosis and inflammation found in the superficial dorsal horn of EAE mice. Reactive glia and inflammatory cells can release a number of mediators, including glutamate, which could increase the excitability and activation of neurons in the spinal cord [37,40]. The high degree of basal activity might also account for the enhanced phosphorylation of NMDA receptor subunits seen in these mice prior to noxious stimulation. This high degree of excitability and activation suggests a likely mechanism for the hypersensitivity/allodynia observed in these animals in response to acutely delivered innocuous stimuli [24].

While changes in the sensitivity of peripheral nociceptors can not be excluded as a potential mechanism mediating these effects, we have previously shown that at this early stage of the disease there are no significant changes in the levels of afferent derived CGRP or galanin in the dorsal horn [24]. It is therefore unlikely that alterations in the content of peripherally derived neuromodulators are mediating these effects.

In contrast, mice with EAE are hypo-responsive following persistent, intense noxious stimulation with formalin. Interestingly, this behavioural profile might also be accounted for by the loss of glutamate transporter expression in these mice. Studies using naïve, adult rats have shown that formalin responses can be significantly attenuated following antisense knockdown of EAAT-2 [21]. It has been postulated that under conditions associated with a rapid increase in glutamate release from primary afferents (such as after formalin stimulation), that blockade or loss of transporter expression may result in an elevation in the levels of extracellular glutamate sufficiently so that it begins to act upon inhibitory pre-synaptic mGluRs [16,21]. It is well established that agonists for pre-synaptic, inhibitory mGluR2/3 receptors can suppress formalin responses and these effects can be reversed by the antagonist LY-341495 [13,32]. In EAE mice, the loss of glutamate transporter expression coupled with the surge of afferent derived glutamate following formalin might lead to elevations in the levels of extracellular glutamate such that these inhibitory receptors are engaged more readily. These processes would suppress glutamate release, decrease spinal activation and inhibit pain processing.

The idea that there is greater engagement of pre-synaptic inhibitory pathways in EAE mice after intense noxious stimulation is supported by our observations that the cellular activation of dorsal horn neurons in response to formalin is unaffected in mice with EAE compared to CFA controls. Additionally, formalin stimulation leads to a significant decrease in the phosphorylation of the NR1 subunit on EAE mice, particularly at the serine 896 site, suggesting that inhibitory mechanisms are being engaged under these conditions. By treating EAE mice with the selective group II mGluR antagonist, LY-341495 prior to formalin stimulation, we were able to restore formalin responses back to control levels. Furthermore, by promoting glutamate transporter activity in EAE mice with MS-153 we find a similar normalization of behaviours. This restoration of pain behaviours suggests that the initial lack of responsiveness to formalin in EAE mice does not arise due to a specific deficit in peripheral afferent input but instead highlights the role of central inhibitory mechanisms in these processes.

Alternatively, the diminished pain behaviours in EAE mice after formalin stimulation may be accounted for by a process of "reverse transport" by the glutamate transporters themselves [26,28]. The proper functioning of the glutamate transporters is dependent on membrane potential and specific transmembrane ion gradients [8,29]. Under pathological conditions, metabolic insults can disrupt intracellular energy stores and perturb ionic gradients such that the transporters begin to "reverse transport" glutamate out into the extracellular space [28]. Instead of absorbing excess extracellular glutamate, the transporters can become a primary source for elevating ambient glutamate levels. This phenomenon has been documented in cases of brain ischemia and in the acute phase after spinal cord injury [17,28]. Interestingly, it has been observed that the inflammatory insults that are the hallmarks of MS and EAE are also associated with mitochondrial dysfunction, energy failure and altered ion exchange mechanisms [9]. These metabolic insults may therefore trigger a similar inverse operation of the transporters in MS and EAE. If ambient glutamate levels were elevated through these processes this could account for the allodynia and ongoing cellular activity observed in EAE mice in the absence of intense noxious stimulation. On the other hand, formalin injection itself may also be capable of triggering "reverse transport" of glutamate [36]. In EAE mice, this could have an additive effect and elevate glutamate levels beyond a threshold that results in the recruitment of pre-synaptic inhibitory mGluRs and thus an attenuation of pain behaviours.

5. Conclusions

In summary, we now provide evidence that decreased expression of spinal glutamate transporters is an important mechanism contributing to the altered pain sensitivity of mice with EAE. Dysregulated glutamate transporter function in these mice can account for the impaired behavioural responses to formalin stimulation and also sheds light on a potential mechanism mediating the allodynia observed in this model. Given the importance of glutamate in pain processing, the glutamate transporters may represent a novel pathway in which to manipulate activity at glutamate receptor complexes, control spinal excitability and manage neuropathic pain.

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Changes in nociceptive sensitivity and object recognition in experimental autoimmune encephalomyelitis (EAE)

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ABSTRACT

Multiple sclerosis is associated with a high incidence of depression, cognitive impairments and neuropathic pain. Previously, we demonstrated that tactile allodynia is present at disease onset in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). We have now monitored changes in object recognition in mice with EAE to determine if altered nociceptive sensitivity is also associated with behavioral signs indicative of cognitive impairment in this model. At the onset of clinical signs, mice with EAE showed impairments in the novel object recognition (NOR) assay, indicative of deficits in cognitive functioning early in the disease course. At the spinal level, we found increased gene expression for the cytokines IL-1β, IL-6 and the glutamate transporter EAAT-2 that coincide with increased nociceptive sensitivity and deficits in object recognition. Increased levels of EAAT-2 mRNA appear to be a response to perturbed protein levels of the transporter as we found a loss of EAAT-2 protein levels in the spinal cord of EAE mice. To determine if changes in the levels of EAAT-2 were responsible for the observed changes in nociceptive sensitivity and cognitive deficits, we treated EAE mice with the β-lactam antibiotic ceftriaxone, an agent known to increase glutamate transporter levels *in vivo*. Ceftriaxone prevented tactile hypersensitivity and normalized performance in the NOR assay in EAE mice. These findings highlight the important interrelationship between pain and cognitive function in the disease and suggest that targeting spinally mediated pain hypersensitivity is a novel therapeutic avenue to treat impairments in other higher order cortical processes.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that leads to severe neurological disabilities. MS is also associated with a high incidence of cognitive impairments and neuropathic pain (He et al., 2011; Osterberg et al., 2005). Central neuropathic pain (CNP), which is thought to arise from lesions or a dysfunction in the CNS, affects an estimated one third of MS patients who report pain as a major symptom of the disease(Svendsen et al., 2003, 2005). An estimated 40% to 60% of MS patients also have cognitive deficits, the most frequent being disorders of memory (He et al., 2000; Jongen et al., 2012). Disturbed memory processing can be detected in the early stages of MS and has been found to progress over time in all disease subtypes (He et al., 2011; Mandolesi et al., 2010). Although both CNP and cognitive impairments are identified in the literature as prominent symptoms reported by MS patients, little is understood about their underlying cellular mechanisms. Currently, effective treatment options are not readily available for these aspects of the disease.

The animal model experimental autoimmune encephalomyelitis (EAE) is a well-established model that has been used to study the pathophysiology of MS for over fifty years (Owens and Sriram, 1995). Despite this, it has only been recently that the model has been used to characterize changes in pain sensitivity in the disease (Musgrave et al., 2011a, 2011b; Olechowski et al., 2009). Previously, we have demonstrated that neuropathic pain behaviors are present before and at disease onset in the $MOG_{35–55}$ mouse model of EAE (Olechowski et al., 2009). We have also reported that changes in pain sensitivity to a persistent noxious stimulus are a result of decreased expression of the EAAT-2 glutamate transporter (Olechowski et al., 2010). Changes in acute pain behaviors in an EAE rat model have also been shown to be associated with decreased glutamate transporter expression (Ramos et al., 2010). Dysregulation of glutamate transporter activity is associated with a persistent elevation in ambient glutamate and has been implicated as a potential mechanism for central sensitization and neuropathic pain following peripheral nerve injury (Sung et al., 2003).

In clinical populations, there is a strong association between chronic pain and cognitive dysfunction. However, studies using the EAE model to examine changes in cognitive function are lacking. To address this,

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we have carried out a series of experiments to further explore the relationship between changes in nociceptive sensitivity and cognitive function in mice with EAE. We have monitored changes in cognitive ability using a standard behavioral assay for object recognition to determine if altered pain sensitivity is also associated with behavioral signs indicative of cognitive impairment in EAE. In addition, we sought to determine whether a common underlying mechanism could mediate these changes in behavior in mice with EAE. We show here that dysregulated expression at the spinal level of the glutamate transporter subtype EAAT-2 is a key mechanism mediating these changes and that promoting EAAT-2 expression using the β-lactam antibiotic ceftriaxone (Rothstein et al., 2005) can normalize nociceptive sensitivity and object recognition in EAE.

Methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with the approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta.

Induction of EAE

A total of 50, 10–12 week old female C57BL/6 mice were used.

Mice were purchased from Charles River Canada (Saint-Constant, Quebec), housed in standard laboratory cages and had free access to food and water throughout the study period. We induced EAE in mice with myelin oligodendrocyte glycoprotein 35–55. (MEVGWYRSPFSRVVHLYRNGK; MOG35–55) obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB). EAE was induced by subcutaneous immunization with 50 μg of $MOG_{35–55}$ emulsified in complete Freund's adjuvant (CFA), (Sigma-Aldrich, Oakville, ON). CFA was supplemented with an additional 5-mg/ml heat killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, BD Biosciences) to a final concentration of 6 mg/ml. The final concentration of CFA in the emulsion was 1.5 mg/ml. An intraperitoneal (I.P.) injection of 300 ng Pertussis toxin (*Bordatella pertussis*) (Sigma-Aldrich, Oakville, ON) was administered at the time of induction and again 48 h later (n = 30). Control mice were treated with CFA as above and Pertussis toxin alone (n = 20).

EAE assessment

All behavioral assessment was conducted by an experimenter who was blind to the experimental grouping. As reported previously (Olechowski et al., 2009, 2010) mice were monitored daily and the clinical signs of EAE were graded on the following scale: Grade 0, normal mouse; Grade 1 (disease onset), flaccid tail; Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; and Grade 4, hindlimb paralysis in one hindlimb or both.

Behavioral testing

Mechanical allodynia

A set of calibrated von Frey hair monofilaments were used to assess the sensitivity to punctate mechanical stimuli. Mice were placed in clear plexiglass chambers on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hindpaw in the ascending order of bending force (range: 0.04–2.0 g). Each hair was applied 5 times per paw, and the number of nociceptive responses (vigorous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The monofilament which produced nociceptive responses greater than 60% of the time was taken as the “threshold”.

Novel object recognition (NOR) test

Mice are first presented with two identical objects (test 1) in an open field (width: 29 cm; length: 44 cm; height: 17 cm); time spent exploring each object was monitored over 5 min using a standard stopwatch. A second test (test 2) was conducted 4 h later where one object was replaced with a novel object. The time spent exploring each object was observed and recorded for 5 min. The difference in time between the two objects was calculated and then averaged for test (i.e. test 1: A–A; test 2 B–A). As a measure of total exploratory activity, the percent of time the mice spent interacting with the objects was calculated. The total amount of time spent with either of the objects over the course of the observation period in each test was divided by the total time of the observation period.

General activity/attention score

Mice were observed in the open field (width: 29 cm; length: 44 cm; height: 17 cm) for a period of 4 min. The activity/attention score is a categorical score given each minute to a mouse based on the duration it spends in a specific sedentary posture. This posture is defined as having both forepaws on the ground and with the head being relatively still with a steady gaze directed below the horizontal (i.e., a head position with the nose pointing towards the floor of the observation box). As previously reported, this score was developed because, while all mice spend considerable amounts of time in one quadrant of the open field, EAE mice were often observed to adopt this posture (Musgrave et al., 2011a, 2011b). These postures are never observed in naive animals and only rarely in control mice immunized with CFA alone. The total duration spent in this posture was measured using a standard stopwatch and scores were given each minute according to the following criteria: 0, mouse is still and with a floor-directed gaze for 45 or more seconds in 1 min; 1, mouse is still and with a floor-directed gaze for 30–45 s in 1 min; 2, mouse is still and with a floor-directed gaze for 15–30 s in 1 min; 3, mouse is still and with a floor-directed gaze for 0–15 s in 1 min. These scores were totaled for each mouse at the end of the 4-min open field trial. Timing was stopped if the mouse interrupted this posture for any reason, including extensions of the head/neck forward or upward (usually to sniff at the air); large, lateral investigatory head movements, or initiation of grooming. A score of 12 is representative of maximal activity/attention during the test period.

Rotarod assay

As a test of gross locomotor ability and coordination, mice were placed on a rotarod at a fixed rotational speed of 16 rpm. Mice were placed on the rotarod and the latency to fall off was determined. The maximum time for a trial was set at 180 s. Mice were trained on the rotarod for two consecutive days prior to disease induction to become familiar with the task. After disease induction, mice were tested on alternate days. Each mouse had three trials and the mean latency to fall per trial was calculated.

Ceftriaxone treatment

Cohorts of mice were treated with the β-lactam antibiotic ceftriaxone (Sigma-Aldrich, Oakville, ON), a compound known to be capable of upregulating glutamate transporter expression in the CNS (Rothstein et al., 2005). Mice were treated with 200 mg/kg I.P. daily starting 7 days after EAE induction (CFA + ceftriaxone n = 5, EAE + ceftriaxone n = 10). Vehicle controls received daily injections of saline starting at 7 days after EAE induction (CFA + vehicle n = 5, EAE + vehicle n = 10).

Real-time RT-PCR

RNA was isolated from spinal cords using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) after lysis with TRIzol (Invitrogen) using the manufacturer's guidelines. RNA dissolved in deionized,

diethylpyrocarbonate-treated water was used for cDNA synthesis. The primers used in real-time PCR were: EAAT-2 fwd 5′-TGA ACG AGG CCC CTG AAG AAA CTA-3′ rev 5′-ATG CCC CCG TGA ATG ATG AGG-3′, IL-6 fwd 5′-ATG GAT GCT ACC AAA CTG GAT-3′ rev 5′-TGA AGG ACT CTG GCT TTG TCT-3′, IL-1β fwd 5′-ACA GAT GAA GTG CTC CTT CCA-3′ rev 5′-GTC GGA GAT TCG TAG CTG GAT-3′, and GAPDH fwd 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ rev 5′-CGG AGT CAA CGG ATT TGG TCG-3′. Semiquantitative analyses were performed by monitoring the increase in the fluorescence of the SYBR-green dye (Invitrogen) in real time on a Bio-Rad (Hercules, CA, USA) i-Cycler. Real-time fluorescence measurements were performed, and at threshold cycle value for each gene of interest. All data were normalized to GAPDH mRNA levels for mice.

Western blots

Mice were anesthetized and euthanized by transcardiac perfusion with 0.9% saline. The brain and lumbar enlargement of the spinal cord were removed and frozen on liquid nitrogen. The brain was further subdivided into a section for the brain that contained the hippocampus with some overlying cortex.

Spinal cord samples

Protein samples (20 μl) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked in 5% milk in PBS-Tween 20 (PBS-T) (0.05%), and then incubated overnight at 4 °C in primary antibodies EAAT-2 (1:500, Santa Cruz Biotechnology), pNR1Ser896 (1:1000, Cedarlane Laboratories) diluted in PBS-T. The membranes were then washed in PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit; Jackson ImmunoResearch) diluted at 1:50,000. The membranes were washed and binding of HRP-conjugated secondary antibodies detected using chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer). Membranes were re-probed with monoclonal mouse anti-β-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

Brain samples

Protein samples (37 μl) were separated on a 10% SDS gel. The samples were transferred onto PVDF membranes (Bio-Rad) overnight at 4 °C. The membranes were blocked in 10% milk in TTBS and incubated overnight at 4 °C in primary antibody EAAT-2 (1:1000, Santa Cruz Biotechnology) in diluted TTBS. The membranes were then washed in TTBS and incubated with HRP-conjugated secondary antibodies (goat anti-rabbit: Jackson ImmunoResearch) diluted at 1:2000. The membranes, similar to spinal cord samples, were washed and binding of HRP-conjugated secondary antibodies detected using chemiluminescence (Amersham ECL Western Blotting Analysis System, GE Heathcare). Membranes were re-probed with monoclonal mouse

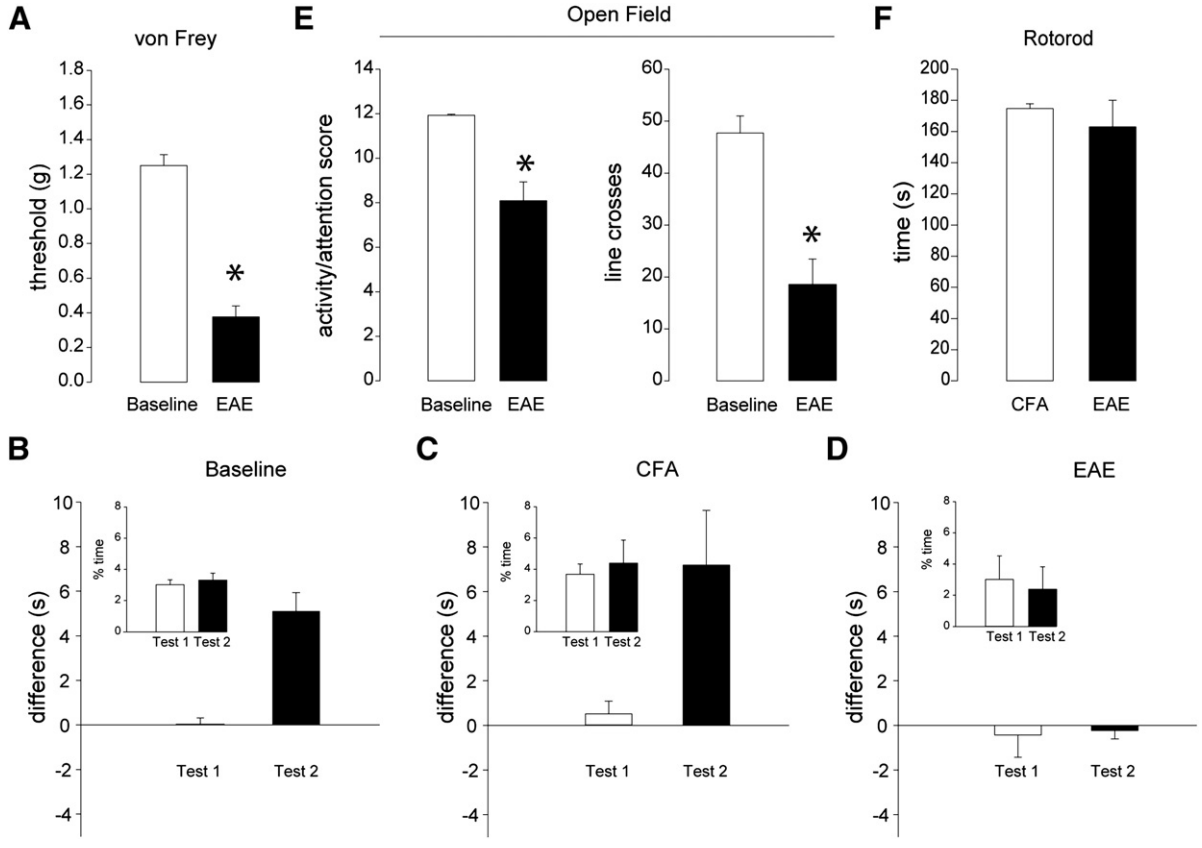

Fig. 1. *The changes in nociceptive sensitivity and impairment in the novel object recognition test in EAE mice at disease onset.* (A) EAE mice exhibit a significant increase in the sensitivity to mechanical force (tactile allodynia) at disease onset (clinical grade 1) when compared to baseline threshold responses (*P<0.01, *t*-test. n = 10). (B) All mice at baseline (prior to disease induction) spend a greater amount of time with the novel object in test 2. (C) CFA control mice matched for day of disease onset with EAE spend a greater amount of time with the novel object, similar to mice at baseline (n = 15). (D) EAE mice do not show a preference for the novel object during test 2 (n = 10). Inset graphs in B–D represent the percentage of total time spent with either object during each test. This is a measure of exploratory behavior. Mice with EAE mice have a slight reduction in their exploratory behavior. (E) The activity/attention score (left panel), which measures how mice attend and interact with their surroundings is significantly reduced in EAE mice compared to baseline measures at disease onset (clinical grade 1) (*P<0.001, Mann–Whitney Rank Sum Test, n = 10). Line crossing in the open field (right hand panel) is also significantly reduced in EAE mice compared to baseline values (*P<0.001, *t*-test, n = 10). (F) Gross locomotor ability in the rotarod assay at disease onset (clinical grade 1) is not impaired in any cohort of EAE mice compared to CFA control mice (P > 0.05, *t*-test).

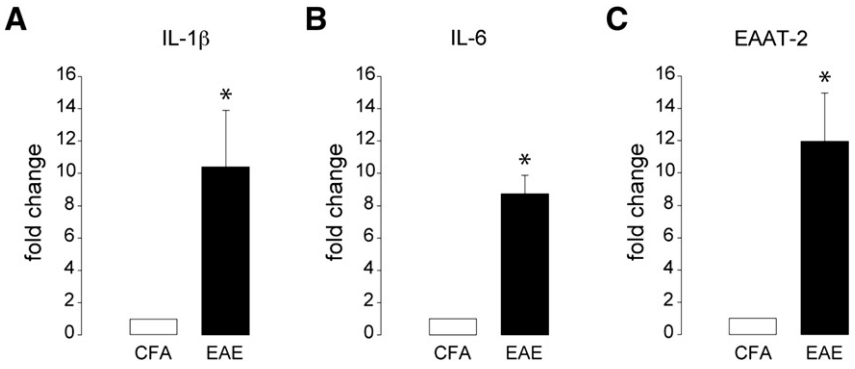

Fig. 2. *IL-1β, IL-6 and EAAT-2 gene expression is increased in EAE mice at disease onset.* (A) IL-1β mRNA levels are upregulated in the spinal cords of EAE mice compared to controls treated with CFA alone (*P = 0.03, *t*-test). (B) IL-6 mRNA levels are upregulated in the spinal cord of EAE mice when compared to control levels from CFA treated mice (P<0.001, *t*-test). (C) EAAT-2 mRNA levels are significantly increased in all EAE cohorts when compared to CFA controls (*P = 0.01, *t*-test) (CFA n = 6, EAE n = 5).

anti-β-actin (1:2000, Sigma) to ensure equal loading of samples. The films were scanned and the changes in protein expression levels between CFA and EAE mice as a ratio were quantified using ImageJ software.

Statistical analysis

Statistical analysis was carried out using the Student's *t*-test and one-way ANOVA with Tukey and Dunnett's post hoc tests. Mann–Whitney rank Sum test and Kruskall–Wallis ANOVA on ranks were used for nonparametric data sets. Significance was set at P<0.05.

Results

Changes in nociceptive sensitivity are associated with impaired object recognition in EAE mice

We have previously reported that mice with EAE develop a robust mechanical allodynia prior to and at the onset of neurological deficits (Olechowski et al., 2009). We replicated this finding by assessing mechanical sensitivity in mice with EAE at disease onset (clinical grade 1). EAE mice exhibit a significant decrease in withdrawal thresholds to punctate mechanical stimulation when compared to baseline measures (Fig. 1A) (P<0.01, *t*-test).

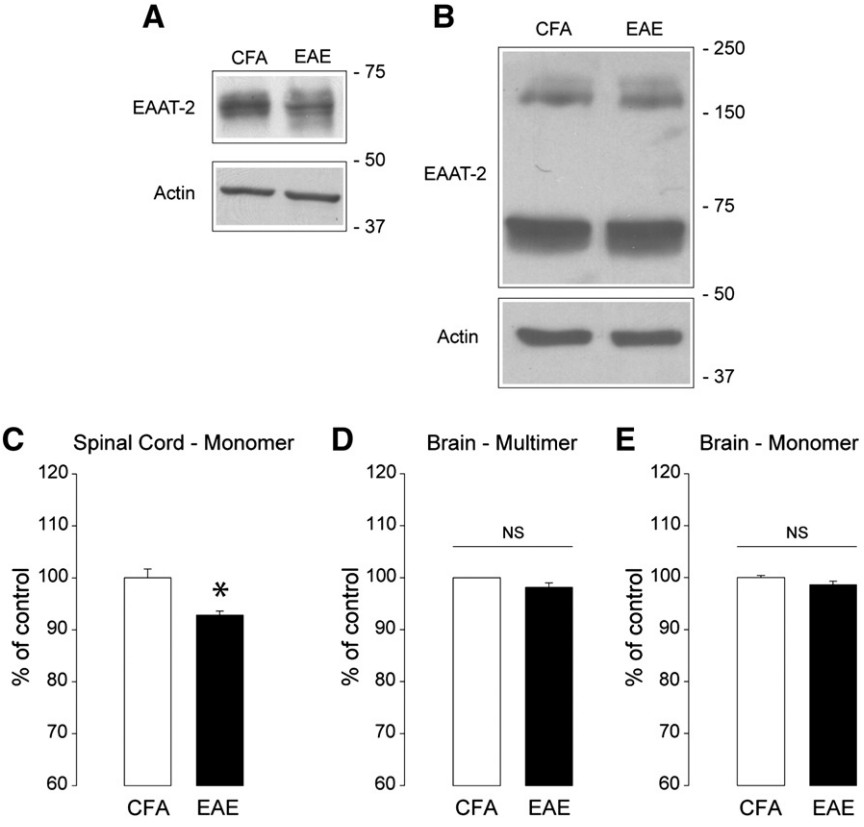

Fig. 3. *Decreased expression of the glutamate transporter EAAT-2 in spinal cords of EAE mice.* (A) There is a significant decrease in the levels of spinal EAAT-2 mice with EAE when compared to CFA. All samples are taken at disease onset (clinical grade 1). (B) There is no difference in EAAT-2 levels (multimer or monomer) in the brains of EAE mice when compared to CFA controls. (C) Quantification of Western blots from the spinal cord for EAAT-2 normalized to beta actin and expressed as a percentage of CFA control levels (*P = 0.01, *t*-test). (D and E) Quantification of the brain EAAT-2 multimer and monomer expression in Western blots normalized to beta actin and expressed as a percent of CFA control levels (P > 0.05, *t*-test) (CFA n = 3; EAE n = 3) (NS = non-significant).

In addition to the changes in pain sensitivity, deficits in cognitive function, particularly memory, are common in patients with multiple sclerosis (MS) (He et al., 2011). Using the novel object recognition (NOR) assay as a test of cognitive ability, we next determined if similar symptoms could be detected in mice with MOG_{35-55} EAE. Prior to disease induction, all mice could discriminate a novel object presented to them in the open field. In 'test 1', the mice are presented with two identical objects and the difference in time spent with each object is around zero seconds. In 'test 2', one of the objects is replaced by a novel object of distinct shape and size and the mice spend greater time exploring and interacting with the novel object (Fig. 1B). Mice immunized with the CFA adjuvant alone show no impairments in the assay (Fig. 1C). For CFA treated mice, the difference in time spent with the novel object compared to the familiar object in test 2 is similar to the baseline condition. In contrast, novel object recognition was significantly impaired in mice with EAE at the onset of disease (clinical grade 1). EAE mice did not show signs of discrimination between the novel and familiar object in test 2 (test 2 mean difference scores: baseline: 5.1 ± 0.84 s; CFA: 7.1 ± 2.4 s; EAE: −0.2 ± 0.32 s) (Figs. 1B, C and D).

To determine if the observed deficits in the NOR assay are due to motivational factors such as reduced overall exploratory behaviors, we calculated the percentage of time the mice spent interacting with the objects during each test. In general, mice with EAE spend slightly less overall time interacting and exploring the objects in test 1 and test 2 compared to baseline levels and CFA treated mice (Fig. 1, inset graphs B–D). Given this decrease in exploratory behavior in the NOR assay, we also assessed signs of sickness behavior in EAE mice at disease onset using a general activity/attention score (Musgrave et al., 2011a, 2011b). Measuring discrete postural changes can serve as adjunct measures for how the mice attend to and interact with their environment. Decreased interaction and/or attention to the surrounding environment is a characteristic feature of sickness behavior. As previously reported, prior to disease induction, healthy mice rarely score below 10–11 using this rating system, meaning that they are consistently paying attention to their surrounding environment (Musgrave et al., 2011a, 2011b). At disease onset (clinical grade 1), EAE mice had significantly lower activity/attention scores compared to baseline measures (baseline median activity/attention score: 12; EAE 'onset': 8) ($P<0.001$, Mann–Whitney Rank Sum Test) (Fig. 1E, left panel). We also monitored exploratory behavior by monitoring the number of line crosses the mice made during the open field observation period. EAE mice at the onset of the disease (clinical grade 1) made significantly fewer line crosses compared to their baseline values prior to disease induction (baseline line crosses: 47 ± 3.3; EAE 'onset': 18 ± 4.9) ($P<0.001$, *t*-test) (Fig. 1E, right panel).

To determine if the reductions in the general activity/attention score and the lack of exploratory behavior was due to impairments in locomotor function in mice with EAE at the onset of the disease (clinical grade 1), gross locomotor ability was assessed using a fixed-speed rotarod assay. At disease onset, when the NOR assay and open field behaviors were assessed, rotarod ability was not significantly affected in EAE mice at clinical grade 1 compared to CFA treated controls (CFA: 174 ± 3s; EAE onset: 163 ± 17s) ($P>0.05$ *t*-test) (Fig. 1F).

IL-1β, IL-6 and EAAT-2 mRNA are upregulated in the spinal cord during EAE

To determine the molecular mechanisms that mediate these changes in behavior in EAE mice, we began by looking at mRNA levels of the pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and the glutamate transporter EAAT-2. We have previously shown that altered levels of EAAT-2 protein in the spinal cord of mice with EAE can affect pain behaviors (Olechowski et al., 2010) and other groups have implicated the involvement of glutamate transporter dysfunction in the generation of pain hypersensitivity in rat models of EAE (Ramos et al., 2010). Both IL-1β and IL-6 can regulate EAAT-2 levels (Okada et al., 2005; Sulkowski et al., 2009) and increased levels of IL-6 and EAAT-2 mRNA have been reported in other EAE models (Mitosek-Szewczyk et al., 2008; Wang et al., 2000). We found that the levels of IL-1β mRNA were elevated in EAE mice at disease onset (Fig. 2A) ($P=0.03$, *t*-test). In addition, both IL-6 and EAAT-2 also showed an increase in mRNA levels in EAE mice at disease onset when compared to CFA control spinal cord samples (Figs. 2B and C) ($P<0.001$; $P=0.01$, t-tests).

Diminished glutamate transporter expression in the spinal cord of EAE mice at disease onset

Given the importance of the glutamate transporters (especially EAAT-2) in regulating excitatory signaling in the CNS (Danbolt, 2001), we next assessed the levels of the EAAT-2 protein in the spinal cord from EAE mice using Western blots. As we have demonstrated previously (Olechowski et al., 2010), compared to CFA controls, mice with EAE mice have reduced levels of the major glutamate transporter EAAT-2 at disease onset ($P=0.01$, *t*-test) (Figs. 3A and C). However, given the deficits in the ability of EAE mice to identify a novel object in the NOR assay, we also assessed the levels of EAAT-2 in the hippocampus/forebrain as a potential mechanism contributing to this decrease in cognitive ability. At this early stage of the disease however, we could not detect significant changes in the levels of EAAT-2 in the brains of mice with EAE when compared to controls treated with CFA alone ($P>0.05$, *t*-test) (Figs. 3B, D and E).

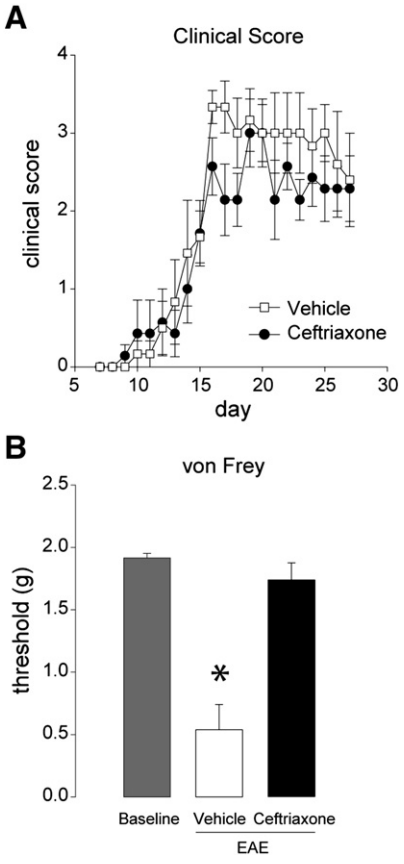

Fig. 4. *Ceftriaxone prevents tactile allodynia in EAE mice.* (A) Daily treatment with ceftriaxone (200 mg/kg) starting at day 7 after EAE induction does not change the disease course of EAE when compared to vehicle treated controls. (B) Ceftriaxone treatment prevents tactile allodynia at disease onset (clinical grade 1) in EAE mice when compared to vehicle treated EAE mice. von Frey thresholds in EAE-vehicle mice are significantly reduced compared to baseline (*$P<0.05$ one-way ANOVA on ranks, Dunn's post hoc test) (EAE + vehicle n = 10; EAE + ceftriaxone n = 10).

Ceftriaxone attenuates tactile hypersensitivity and cognitive deficits in EAE

To determine whether decreased levels of spinal EAAT-2 are involved in generating nociceptive hypersensitivity and cognitive deficits in the MOG_{35-55} EAE model, we treated EAE mice with ceftriaxone, a β-lactam antibiotic that has the ability to upregulate EAAT-2 in the CNS (Ramos et al., 2010; Rothstein et al., 2005). We first assessed how ceftriaxone treatment affected the clinical signs of the disease. Daily treatment with ceftriaxone (200 mg/kg) (Melzer et al., 2008) beginning seven days after disease induction had no effect on the disease course compared to vehicle treated EAE mice (Fig. 4A). However, daily treatment with ceftriaxone attenuated the tactile hypersensitivity that is present at disease onset ($P<0.05$ one-way ANOVA on ranks, Dunn's post hoc test) (Fig. 4B).

Daily ceftriaxone treatment also improved performance in the NOR assay. Prior to the start of treatment with either ceftriaxone or vehicle, all mice induced with EAE had impaired novel object recognition in test 2 compared to controls treated with CFA alone (test 2 mean difference scores: baseline: 9.1 ± 0.32 s; CFA day 7: 13.2 ± 4.1 s; EAE day 7: 2.5 ± 0.9 s) (Fig. 5A, i, ii and iii). EAE mice then began daily treatment with either ceftriaxone or vehicle and were re-assessed in the NOR assay when the first clinical signs of the disease appeared ('onset', clinical grade 1). Object recognition remained impaired in vehicle treated EAE mice compared to CFA treated mice tested on the same day (test 2 mean difference scores: CFA matched for 'onset': 17.5 ± 2.8 s; EAE + vehicle at 'onset': 1.2 ± 0.7 s) (Fig. 5B, i and ii). In contrast, EAE mice treated with daily ceftriaxone had improved discrimination of the novel object in test 2 with performance approaching pre-disease levels (test 2 mean difference score: EAE + ceftriaxone at 'onset': 9.4 ± 3.7) (Fig. 5B iii). Interestingly, exploratory behavior remained significantly lower in both groups of EAE mice compared to CFA controls (inset graphs Fig. 5B). In addition, we found that the activity/attention scores of EAE mice treated with daily ceftriaxone were not significantly different from vehicle treated controls. Compared to baseline values, both groups of EAE mice had significantly reduced activity/attention scores at disease onset (baseline median activity/attention score: 12; EAE + vehicle 'onset': 5; EAE + ceftriaxone 'onset': 5) ($P=0.001$, Kruskal–Wallis One Way ANOVA on ranks, Dunn's method) (Fig. 6A). However, gross locomotor ability was not affected at this stage of the disease. As shown previously, rotarod performance is not significantly affected in either group of EAE mice at the onset of disease (clinical grade 1) when testing takes place (Fig. 6B).

Ceftriaxone treatment increases EAAT-2 expression and reduces NMDA receptor phosphorylation in the spinal cord of mice EAE

To determine if ceftriaxone was increasing the levels of spinal EAAT-2 in mice with EAE, EAAT-2 levels were assessed using Western blots at disease onset. The significant reduction in spinal EAAT-2 levels in vehicle treated EAE mice was prevented in mice treated with daily ceftriaxone (Figs. 7A and C). A consequence of dysregulated EAAT-2 expression is an increased activation of excitatory glutamate receptors. We have previously shown that EAE mice have an increase in the levels of phosphorylated NR1 NMDA receptor subunits at the spinal level (Olechowski et al., 2010). Increased phosphorylation of NR1 (pNR1)

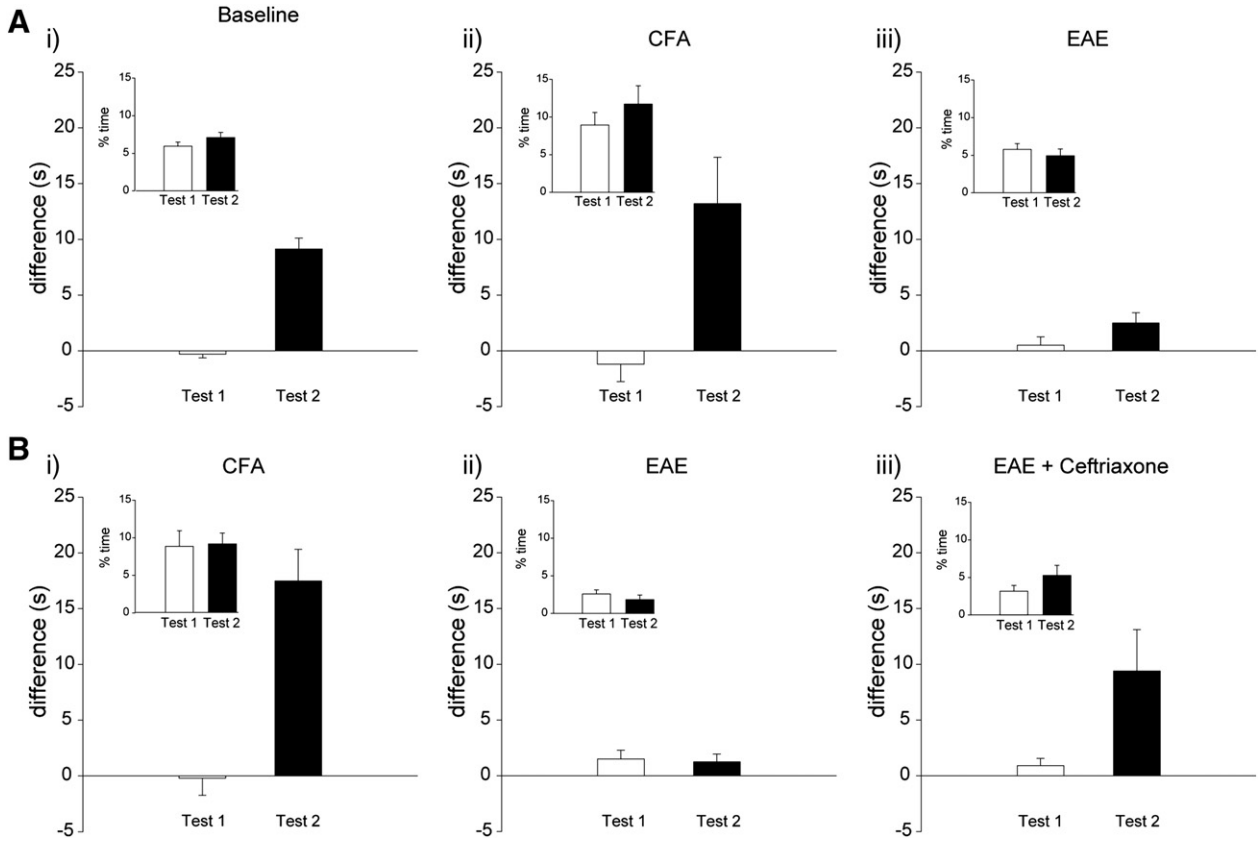

Fig. 5. *Ceftriaxone treatment restores novel object recognition in EAE mice.* (A) (i) At baseline prior to disease induction, all mice spend a greater amount of time with the novel object during test 2. (ii) At day 7 after disease induction, CFA control mice spend a greater amount of time with the novel object during test 2. (iii) At day 7 after disease induction and prior to starting treatment, all EAE mice fail to show a preference for the novel object during test 2. (B) (i) CFA control mice spend a greater amount of time with the novel object during test 2 when tested at matched timepoints with EAE mice that reach the onset of disease (clinical grade 1). (ii) At disease onset (clinical grade 1), vehicle treated EAE mice continue to show impairments in their ability to distinguish the novel object during test 2. (iii) At disease onset (clinical grade 1), ceftriaxone treated EAE mice spend a greater amount of time with the novel object during test 2. Inset graphs in (A and B) represent the percentage of total time spent with the objects in each test as a measure of exploratory behavior. Exploratory behavior remains decreased in EAE mice relative to CFA control mice regardless of treatment (CFA n = 10; EAE + vehicle n = 10; EAE + ceftriaxone n = 10).

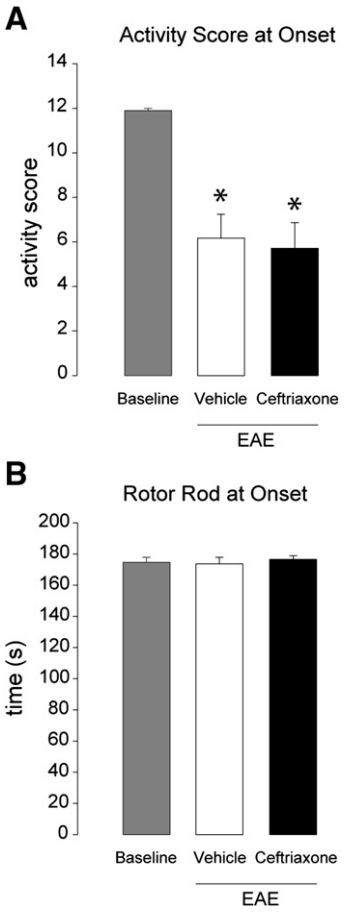

Fig. 6. *Ceftriaxone treatment does not affect activity/attention scores or gross locomotor ability at disease onset.* (A) The activity/attention score is significantly decreased in both the ceftriaxone and vehicle treated EAE mice at disease onset when compared to baseline (*P = 0.001, Kruskal–Wallis One Way ANOVA on ranks, Dunn's method). (B) Locomotor ability as measured with the rotarod test at disease onset (clinical grade 1) is not different from baseline in both EAE groups (P > 0.05, One Way ANOVA).

has been implicated in the generation of nociceptive hypersensitivity (Olechowski et al., 2010). We therefore examined the levels of pNR1 at serine 896 (pNR1^{896}) at disease onset (clinical grade 1) in EAE mice treated with the vehicle or ceftriaxone. Increased levels of spinal pNR1^{896} were seen in both groups of EAE mice compared to controls treated with CFA alone (Fig. 7B). However, pNR1^{896} levels were significantly higher in EAE mice treated with vehicle compared to ceftriaxone treated EAE mice ($P < 0.05$, one-way ANOVA, Tukey post hoc test) (Fig. 7D).

Discussion

Chronic pain and cognitive deficits are now recognized as major symptoms associated with MS (Jongen et al., 2012; Svendsen et al., 2003, 2005). To date however, few studies have examined the underlying causes of these features of the disease, or the relationship between the two using the widely accepted animal model EAE. We have previously reported that nociceptive hypersensitivity develops in the MOG$_{35–55}$ EAE mouse model prior to and at disease onset (Olechowski et al., 2009). We have now characterized nociceptive hypersensitivity in mice with MOG$_{35–55}$ EAE and also examined cognitive function in this model using the novel object recognition assay. Our findings demonstrate that nociceptive hypersensitivity to tactile stimuli and deficits in object recognition occur in tandem and can be observed very early in the disease course. Interestingly, normalizing nociceptive sensitivity in mice with EAE leads to improved performance in the NOR assay, illustrating the important interrelationship between these two variables.

A common feature of both MS and the EAE models is the decrease in the protein levels of the dominant glutamate transporter EAAT-2 (Olechowski et al., 2010; Pampliega et al., 2008; Vercellino et al., 2007). Decreased levels of spinal glutamate transporters are associated with changes in nociceptive thresholds in a variety of peripheral nerve and spinal cord injury models (Tao et al., 2005). This appears to be a common feature of the MOG$_{34–55}$ EAE model as well. Using quantitative RT-PCR, we find that gene expression of the cytokines Il-1β and IL-6 are upregulated in the spinal cord of EAE mice. An 'IL-6 amplifier mechanism' in the lumbar spinal cord has been characterized as a major mediator of EAE disease progression (Arima et al., 2012). Interestingly, IL-6 is also known to be a major factor triggering the downregulation of EAAT-2 (Sulkowski et al., 2009). The observed changes in tactile sensitivity in EAE mice may therefore be accounted for by lumbar IL-6 upregulation and subsequent decreases in the levels EAAT-2 protein in the spinal cord.

In a rat model of EAE, upregulating the levels of EAAT-2 using the β-lactam antibiotic ceftriaxone, reduces the nociceptive hypersensitivity associated with EAE (Ramos et al., 2010). We found that in the MOG$_{35–55}$ mouse EAE model, ceftriaxone is equally effective at normalizing tactile withdrawal thresholds. Ceftriaxone's effects appear to be mediated primarily by changes in EAAT-2 levels in the spinal cord as we found no significant changes in EAAT-2 levels in the brains of EAE mice at the early stages of disease progression when tactile hypersensitivity was most pronounced. By stabilizing EAAT-2 levels in the spinal cord, ceftriaxone treatment is also able to reduce the levels of phosphorylation at the NR1 NMDA receptor subunit, a well established pathway leading to pain hypersensitivity in a number of animal models including EAE (Olechowski et al., 2010; Zou et al., 2000). These results confirm ceftriaxone's ability to upregulate or stabilize the levels of EAAT-2 in the spinal cord of the MOG$_{35–55}$ EAE model and suggest that the decreased pain hypersensitivity and concomitant improvement in the NOR assay in ceftriaxone treated mice may arise as a result of reduced excitatory drive to spinal glutamate receptors.

Interestingly, we find that deficits in object recognition are also present in mice with MOG$_{35–55}$ induced EAE. One of the most common cognitive deficits assessed in the MS patient population are problems with memory retention (He et al., 2011). Previous studies in both rat and mouse models of EAE reported deficits in spatial recognition using the Morris water maze (D'Intino et al., 2005; Ziehn et al., 2010). In addition, there is evidence that long-term potentiation, a cellular substrate for learning and memory, is impaired in EAE models (Mandolesi et al., 2010). Importantly, the observed deficits of mice with MOG$_{35–55}$ EAE in the NOR assay cannot be accounted for by any significant impairment of gross locomotor ability. At this stage of the disease, performance in the fixed speed rotarod assay is unchanged in EAE mice when NOR testing occurs (disease onset, clinical grade 1). Although deficits in gross locomotor ability do not appear to be a factor for the impaired performance of MOG$_{35–55}$ EAE mice in the NOR assay, a recent study has shown anatomical evidence of neurodegeneration in the visual system of a rat model of EAE prior to the onset of optic neuritis (Fairless et al., 2012). It is unlikely however that the deficits in object recognition behavior in MOG$_{35–55}$ EAE mice can be accounted for by these potential early impairments of visual function as could be inferred from the latter anatomical observations of early onset retinal ganglion cell loss and microglial cell activation in this latter EAE rat model (Fairless et al., 2012). Although the timing of these neurodegenerative events in the retina coincides with the onset of the behavioral deficits reported in MOG$_{35–55}$ EAE mice (Fairless et al., 2012) there are two critical elements that rebut this possibility: 1) we show here that treating mice with ceftriaxone can reverse the changes in NOR performance in EAE mice; and 2) retinal ganglion cell death is irreversible, and this applies to any other

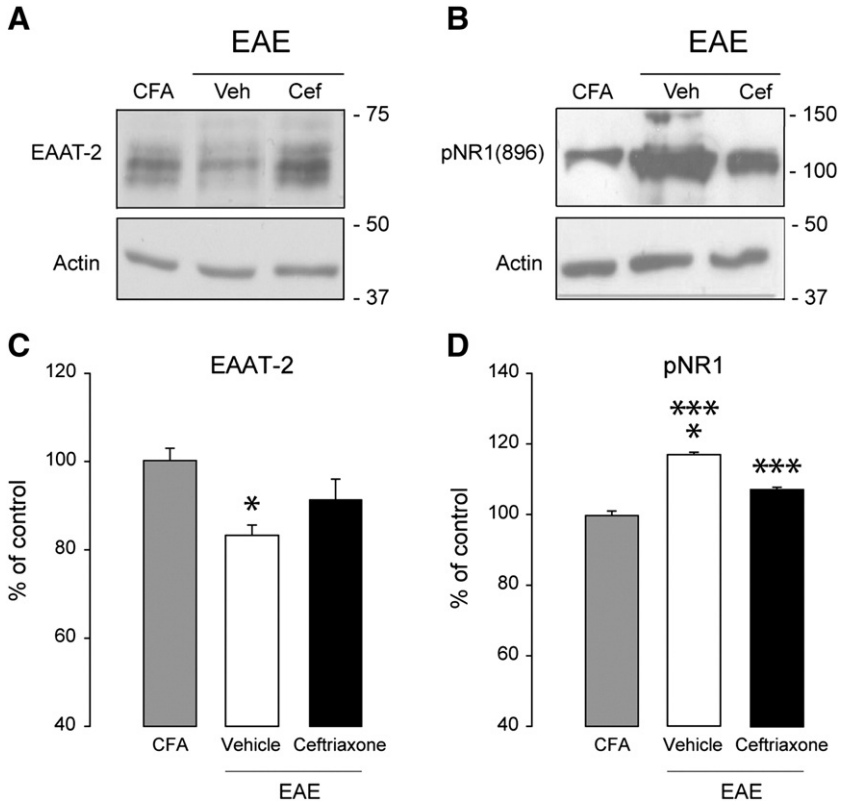

Fig. 7. *Ceftriaxone increases the levels of EAAT-2 and reduces phosphorylation of NR1 serine 896 in EAE mice spinal cords.* (A) EAAT-2 levels are significantly decreased in the spinal cord of vehicle treated EAE mice at disease onset but are normalized in EAE mice treated with ceftriaxone. (B) The levels of pNR1 serine 896 are significantly higher in both groups of EAE mice compared to the levels in CFA controls. However, the levels of $pNR1^{896}$ are significantly lower in the ceftriaxone treated EAE mice when compared to the vehicle treated EAE mice. (C–D) Quantification of the spinal cord Western blots for EAAT-2 (C) and pNR1 serine 896 (D) normalized to beta actin and expressed as a percent of CFA control levels (*P<0.05, one-way ANOVA, Dunnett's post hoc test, C) (***P<0.05, one-way ANOVA, Dunnett's post hoc test, *P<0.05, *t*-test, EAE vehicle vs. EAE ceftriaxone, D) (CFA n = 3; EAE + vehicle n = 4; EAE + ceftriaxone n = 4).

neurodegenerative events in the retina and optic nerve of adult mammals (Sauve and Gaillard, 1995). Furthermore, we have recently begun studies using electrophysiological parameters to assess visually-evoked retinal and cortical responses in mice with $MOG_{35–55}$ EAE two weeks after the onset of clinical signs in the disease (a time point that is much later in the disease progression than we have tested in the current studies). Preliminary results from these studies of visually-evoked retinal and cortical responses indicate that there are no functional visual deficits in EAE mice at all clinical grades (from 1 to 4) compared to CFA treated control mice (Supplemental Fig. 1). This is not surprising given that C57BL/6 mice induced with $MOG_{35–55}$ EAE only begin to show signs of retinal ganglion cell (RGC) death much later in the disease course (Quinn et al., 2011). Therefore, it is implausible that the therapeutic effects of ceftriaxone treatment are the result of neuroprotective or regenerative responses in the retina.

We did however, observe significant reductions in the total exploratory behavior of EAE mice during the NOR testing sessions. The absence of gross locomotor deficits in EAE mice at this early stage of the disease suggests that the decrease in general activity/attention scores and reduced exploratory behavior in the NOR assay are not a consequence of impaired locomotor ability. Instead, these reductions in exploration are likely related to the sickness-like behavior that are well documented in EAE mice (Musgrave et al., 2011a, 2011b; Pollak et al., 2002, 2003). Decreased motivation to explore and interact with the objects due to sickness or pain is therefore more likely the cause for the poor performance in this assay. Indeed, the importance of nociceptive hypersensitivity as a mediator of the deficits in object recognition EAE mice is highlighted by our observations that normalizing tactile withdrawal thresholds in the disease can restore object recognition without any significant change in total exploratory behavior in the test. Taken together, these findings suggest that the beneficial effects of ceftriaxone in the NOR assay are not due to a generalized anti-depressant effect or through improvements in locomotor ability but are likely due to a generalized reduction in nociceptive hypersensitivity.

Conclusions

Taken together our results demonstrate that the $MOG_{35–55}$ EAE model can be a useful tool for studying the mechanisms leading to pain hypersensitivity and cognitive deficits seen in the early stages of MS. We show here that changes in nociceptive sensitivity and deficits in object recognition in the $MOG_{35–55}$ EAE model develop very early in the disease course, even before the emergence of clinical signs. Changes in pro-inflammatory cytokine expression and the concomitant decrease in spinal EAAT-2 expression is a key underlying mechanism mediating these changes in behavior. Our data suggest that effective control of nociceptive sensitivity can have a broad impact on other higher order behaviors such as object recognition. Targeting the glutamate transporter system may represent an important avenue as a potential treatment option for not only abnormal pain sensitivity but also the cognitive deficits associated with the disease.

Conflict of interest statement

The authors have no competing financial interests to declare.

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