Pain and analgesia in experimental autoimmune encephalomyelitis: Contribution of the central nucleus of the amygdala

by

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Abstract

Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation, degeneration and demyelinating lesions within the central nervous system. Chronic pain is a highly prevalent symptom associated with MS, affecting 50-80% of patients over the course of their disease. Unfortunately, MS-related pain responds poorly to classical analgesics, contributing to overall disease burden and reduced quality of life. Despite the high incidence of chronic pain in MS, the underlying mechanisms remain poorly understood.

The amygdala is a small nucleus within the limbic brain that is well-known for its role in integrating emotional and sensory information. The central nucleus of the amygdala, in particular, has recently emerged as an important brain center for pain modulation. Several chronic pain conditions are associated with alterations in amygdala neurocircuitry and anatomy, suggesting that these maladaptive changes contribute to pathological pain. This thesis focuses on elucidating the role of the central amygdala in the pathophysiology of pain in MS.

I employed the myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE) model of MS in male and female C57BL/6 mice. Prior to the onset of clinical symptoms (pre-symptomatic phase), I assessed thermal pain hypersensitivity and opioid-induced analgesia with the tail withdrawal assay, and morphine reward with the conditioned place preference paradigm. Brain and spinal cord tissue were extracted on day of disease onset and prepared for immunohistochemical staining or fluorescent *in situ* hybridization.

In the early stages of disease, male and female EAE mice exhibited robust microglial activation in the central amygdala, which was associated with heat hyperalgesia, impaired morphine reward and loss of morphine antinociception in females. EAE animals also displayed a lack of responsivity within the antinociceptive somatostatin-expressing neurons in the central

amygdala. Induction of focal microglial activation in naïve mice via bilateral stereotactic microinjection of lipopolysaccharide into the central amygdala attenuated morphine analgesia in females, similar to that observed in EAE animals.

Collectively, my data indicate that activated microglia within the central nucleus of the amygdala contribute to the sexually dimorphic effects of morphine and drive neuronal adaptations that lead to pain hypersensitivity in EAE. Therefore, novel approaches to treating pain in MS must take the effects of sex on pain regulation and treatment outcomes into consideration. My work further suggests that inhibiting microglial activation and restoring nociceptive signaling are potential strategies for improving pain management and analgesic efficacy in this patient population.

Preface

This thesis is an original work written by Zoë Dworsky-Fried. The current research project was conducted at the University of Alberta under the supervision and guidance of Dr. Taylor, Dr. Kerr, Dr. Winship and Dr. Webber. The current research project received research ethics approval from the University of Alberta Research Ethics Board. All animal experiments and procedures were conducted in accordance with the Canadian Council on Animal Care's Guidelines and Policies and with protocols approved by the University of Alberta Health Sciences Animal Care and Use Committee (AUP#00000274; AUP#00002493). This research project received funding support from a CIHR Canada Graduate Scholarship.

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List of Abbreviations

ANOVA	Analysis of variance
BBB	Blood-brain barrier
BLA	Basolateral amygdala
CD4	Cluster of differentiation 4
CeA	Central nucleus of the amygdala
CeLC	Laterocapsular region of the central amygdala
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
CPP	Conditioned place preference
DAPI	4',6-diamidino-2-phenylindole
DRG	Dorsal root ganglion
EAE	Experimental autoimmune encephalomyelitis
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
Iba-1	Ionized calcium-binding adapter molecule-1
ITC cells	Intercalated cells
LPS	Lipopolysaccharide
MOG	Myelin oligodendrocyte glycoprotein
MOR	Mu opioid receptor
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NAc	Nucleus accumbens

PAG	Periaqueductal gray
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PFA	Paraformaldehyde
PKC	Protein kinase C
PLP	Proteolipid protein peptide
RVM	Rostral ventromedial medulla
SEM	Standard error of the mean
SNI	Spared nerve injury
SOM	Somatostatin
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
TMEV	Theiler's murine encephalomyelitis virus
TWL	Tail withdrawal latency
VEH	Vehicle

CHAPTER 1: Introduction

1.1 What is pain?

Pain is an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage. Although unpleasant, the sensation of pain is an imperative component of the body's defense system and facilitates survival (Grichnik and Ferrante, 1991). Pain alerts an individual to withdraw from immediate tissue damaging stimuli and prevents further damage to the injury site during the healing process. The critical role of pain is most evident in individuals who have congenital insensitivity to pain, a rare genetic condition that causes an inability to sense noxious or damaging stimuli (Cox et al., 2006). The absence of this normally protective response results in frequent injuries and higher mortality rates early in life (Bennett and Woods, 2014).

When pain persists beyond tissue damage and normal healing time, it is termed chronic pain. Chronic pain is a disease state that is mechanistically distinct from physiological acute pain, encompassing adaptations along the entire neural axis (Tracey and Bushnell, 2009). It can be defined as any pain that lasts or recurs for more than three to six months, and thus lacks the acute warning function of physiological pain (Treede et al., 2015). Chronic pain can arise as a consequence of injuries to the peripheral or central nervous system, or may be caused by a variety of conditions, such as autoimmune disorders, cancer, metabolic diseases, and neurodegenerative diseases (Finnerup et al., 2015). Globally, it is estimated that 20% of the general population suffers from chronic pain, with prevalence increasing with age (Goldberg and McGee, 2011). The experience of chronic pain interferes with all aspects of patients' lives, negatively impacting physical and mental health, social relationships, and daily activities (Dueñas et al., 2016). In addition to the severe clinical burden placed on an individual, the direct and indirect costs associated with chronic pain to the Canadian economy are approximately $7.2

billion per year (Hogan et al., 2016). Given the chronic, non-fatal nature of this condition, chronic pain represents a burgeoning clinical, economic, and sociological burden in the health care setting. Unfortunately, classical analgesics, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids, often provide inadequate relief for chronic pain and are generally coupled with adverse side effects (Yekkirala et al., 2017). Moreover, currently available first-line pharmacotherapies for the treatment of chronic pain, including tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and gabapentinoids, are only modestly efficacious (Finnerup et al., 2015). As such, there is an unmet clinical need to develop novel classes of analgesics with improved efficacy and diminished side effects than those currently available. A greater understanding of the fundamental mechanisms that underlie chronic pain pathophysiology and etiology may reveal novel targets for the management of this condition.

1.1-1 Basic neurobiological mechanisms of pain

Pain transduction and perception are complex, involving fundamental biological events along the neural axis. The sensation of pain is initiated with the activation of nociceptors, a diverse subpopulation of specialized peripheral sensory neurons that respond to damaging or potentially damaging stimuli, such as thermal, mechanical or chemical insults (Dubin and Patapoutian, 2010). Nociceptors can be generally classified as unmyelinated, small-diameter C-fibers or thinly myelinated, medium-diameter Aδ fibers that respond to multiple sensory modalities to convey poorly localized slow pain (C-fibers) and well-localized fast pain (Aδ fibers) (Ringkamp and Meyer, 2008). Primary afferent nociceptors are pseudounipolar neurons with cell bodies located in the dorsal root ganglion (DRG) and peripheral and central branches that project to the periphery and spinal cord, respectively (Basbaum et al., 2009). The axon of the

primary afferent nociceptor transmits the nociceptive message from the periphery to projection neurons within the Rexed laminae of the spinal cord. The spinal cord dorsal horn receives the majority of the primary afferent input (Moehring et al., 2018). Complex spinal cord circuitry allows for the integration and modulation of peripheral sensory information across modalities, such as temperature, touch, and pain (Sandkühler, 2009). Nociceptive information is transmitted to higher brain centers via the ascending spinal tracts, which terminate within the periaqueductal gray (PAG), parabrachial nucleus, and thalamus (Basbaum et al., 2009). Third-order neurons carry information from the thalamus to the somatosensory cortex, allowing for the localization of pain and the discrimination of pain intensity (Basbaum et al., 2009). Other projection neurons engage the limbic system via connections in the brainstem and amygdala, contributing to the affective component of the pain experience, such as the aversive and unpleasant nature of a noxious stimulus (Stucky et al., 2001; Price et al., 2006; Basbaum et al., 2009).

1.1-2 The transition from acute to chronic pain

We now appreciate that the sensation of pain is a multidimensional experience that encompasses sensory, affective, and cognitive elements; the interplay between these factors can amplify or diminish the perception and experience of pain. Both functional and structural alterations within pain pathways can lead to the maladaptive transition from acute to chronic pain, resulting in the development of a chronic clinical condition. A growing body of evidence demonstrates that the anatomy and physiology of the brain in chronic pain is distinct from that of individuals experiencing acute pain (Apkarian et al., 2011). Although not yet fully understood, considerable progress has been made in understanding the basic cellular and molecular mechanisms that contribute to the chronicity and intractability of pain.

1.1-2.1 Corticolimbic circuitry

Traditionally focused on the sensory system, peripheral sensitization in primary sensory neurons of DRG and trigeminal ganglia, as well as central sensitization of pain-processing neurons in the spinal cord and brain, were believed to be driving the chronic pain condition (Basbaum et al., 2009; Gold and Gebhart, 2010; Woolf, 2011). Recent evidence, however, indicate that adaptions in brain regions involved in emotional and motivated behaviour (corticolimbic circuitry) are particularly important in the transition from acute to chronic pain. Corticolimbic circuitry is primarily composed of the medial prefrontal cortex, nucleus accumbens (NAc), amygdala, hippocampus, and ventral tegmental area. Neuroimaging studies conducted in humans and animal models of chronic pain demonstrate that structural and functional plasticity within these circuits accompanies the transition from acute to chronic pain (Baliki et al., 2008, 2012; Hashmi et al., 2013; Vachon-Presseau et al., 2016; Chang et al., 2017). In patients with persistent subacute back pain, spontaneous episodes of pain are initially processed in traditional nociceptive brain regions, such as the thalamus and anterior cingulate cortex, and progressively shift toward limbic circuitry once patients transition to chronic pain states (Hashmi et al., 2013). These patients also display a reduction in gray matter density in the insular cortex, primary somatosensory cortex, and NAc (Baliki et al., 2012). Moreover, chronic pain patients show altered functional connectivity and reductions in gray matter volume in the amygdala and hippocampus (Baliki et al., 2006; Mutso et al., 2012; Hashmi et al., 2013). Consistent morphological and functional abnormalities in corticolimbic regions are also observed in rodent models of chronic pain (Metz et al., 2009; Ji and Neugebauer, 2011; Mutso et al., 2012; Chang et al., 2017).

Given the extensive evidence, it is clear that chronic pain is associated with significant functional and morphological adaptations within corticolimbic circuitry that drive the transition from acute to chronic pain. In addition, deficits in corticolimbic function are associated with negative hedonic states and may contribute to the comorbid mood disorders that are highly prevalent within the chronic pain population (Elman et al., 2013). Interventions aimed at restoring function within this system may therefore be effective in improving chronic pain, as well as alleviate negative affective symptoms.

1.1-2.2 Neuroinflammation

Pain is classically viewed as being mediated solely by neuronal mechanisms. In recent years, the significant contribution of glial cells and neuron-glial interactions in the peripheral and central nervous systems to the initiation and maintenance of chronic pain has become increasingly evident. Microglia are the resident macrophage cells of the CNS that originate from bone marrow-derived monocytes and migrate during perinatal development. Microglia make up approximately 5-15% of total cells in the human brain and play a critical role in CNS maturation, neuronal homeostasis, and host defense (Pelvig et al., 2008). Under normal, physiological conditions, microglia actively sense the neural parenchyma through extension and retraction of their motile processes to modulate development and brain homeostasis (Nimmerjahn et al., 2005). In response to pathological stimuli, such as infection, trauma, altered neuronal function, or disease, microglia become activated and undergo a series of morphological and functional changes. Activated microglial cells initiate a variety of innate defense mechanisms, including phagocytosis of toxic debris, antigen processing and presentation, and release of a myriad of cytokines, chemokines and neurotrophic factors (Hanisch, 2002; Walter and Neumann, 2009).

Aberrant activation of microglia has been reported to produce detrimental effects on the CNS, through releasing reactive nitrogen and oxygen species that can damage brain epithelial cells and compromise the blood-brain barrier (BBB), thereby contributing to disease pathogenesis (Kacimi et al., 2011; Bachiller et al., 2018).

There is mounting evidence that spinal cord microglia are critically involved in the development of exaggerated pain states of diverse etiologies (Svensson et al., 2003; Coull et al., 2005; Tozaki-Saitoh et al., 2008; Inoue and Tsuda, 2009; Beggs et al., 2012; Sorge et al., 2015). Elevated microglial reactivity and proliferation are consistently observed in animal models of acute, inflammatory, neuropathic, and MS-related pain (Coyle, 1998; Sweitzer et al., 1999; Tanga et al., 2004; Zhang et al., 2005; Zhang and Koninck, 2006; Beggs and Salter, 2007; Olechowski et al., 2009; Zhong et al., 2010). In brief, injured nerve-derived signals induce activation of spinal cord microglia and subsequent production of several pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. This in turn activates and sensitizes pain-transmitting neurons in the dorsal horn of the spinal cord, resulting in central sensitization and enhanced pain transmission (McMahon et al., 2005; Zhang and Koninck, 2006; Beggs et al., 2012; Ferrini and De Koninck, 2013). Intrathecal injection of activated microglia produces tactile allodynia in naïve rats, suggesting that microglial activation is sufficient to induce pain hypersensitivity (Tsuda et al., 2003). Conversely, targeted pharmacological interventions that inhibit the activation (Raghavendra et al., 2003b; Hua et al., 2005; Hains and Waxman, 2006; Taylor et al., 2015) or proliferation (Gu et al., 2016) of microglia attenuate a wide array of pathological pain states, including neuropathic, inflammatory, and postoperative pain. Notably, disruption of microglial activation has no effect on normal responses to acute pain

stimuli, supporting the notion that glia are specifically involved in pathological pain processes (Meller et al., 1994; Watkins et al., 1997; Milligan et al., 2000).

While much of the evidence about the glial contribution to chronic pain emanates from studies in the spinal cord, more recent reports have found widespread pain-induced microglial activation throughout the neural axis (Sawada et al., 2014; Taylor et al., 2015, 2017; Barcelon et al., 2019). Chronic neuropathic pain in rodent models is accompanied by microglial activation in diverse brain regions associated with pain and affect, including the thalamus, medial prefrontal cortex, amygdala, hippocampus, and NAc (Gui et al., 2016; Potter et al., 2016; Taylor et al., 2017; Barcelon et al., 2019). In humans, patients with chronic back pain show microglial activation in several of the same brain regions (Loggia et al., 2015). Collectively, these findings implicate neuroinflammation along the neuroaxis as a key mechanism in the progression of chronic pain phenotypes.

1.1-3 Endogenous and exogenous opioids in pain

Opioids have been used medicinally for the relief of pain and sedation for thousands of years. In the 1970s and 1980s, advancements in neuroscience and medicinal chemistry allowed for the discovery of endogenous opioid peptides and receptors (Corbett et al., 2006). Today, opioid therapy is the mainstay approach for severe acute, perioperative, and chronic pain. As the use of opioids for pain management has increased dramatically in the past twenty years, understanding the neural mechanisms that underlie the effects of opioids in health and disease is necessary to develop novel therapies that dissociate opioid analgesia from deleterious side effects, such as dependence and respiratory depression (Benyamin et al., 2008).

The endogenous opioid system plays a vital role in modulating the experience and management of pain. Three genetically distinct families of opioid peptides are considered classical members of the endogenous opioid system: β-endorphin (derived from the precursor pro-opiomelanocortin), methionine (Met)- and leucine (Leu)-enkephalins (derived from pre-proenkephalin), and dynorphins (derived from pre-prodynorphin). There are three major families of opioid receptors: μ- (MOR), κ- (KOR), and δ-opioid receptors (DOR), which are all seven-transmembrane spanning proteins that couple to inhibitory G proteins (Simon, 1991; Al-Hasani and Bruchas, 2011; Benarroch, 2012). Each receptor is encoded by a unique gene (*OPRM1, OPRK1, OPRD1,* respectively). Activation of opioid receptors by endogenously produced opioid peptides or by exogenously administered opiates, such as morphine or fentanyl, promotes dissociation of inhibitory G_{α} and $G_{\beta\gamma}$ protein subunits. The G_{α} and $G_{\beta\gamma}$ subunits subsequently act on various intracellular effector pathways, resulting in presynaptic inhibition of neurotransmitter release and postsynaptic hyperpolarization (Al-Hasani and Bruchas, 2011; Benarroch, 2012).

Opioid peptides and their receptors have a widespread but distinct expression throughout the nervous system, particularly in circuits involved in nociceptive modulation, reward, stress response, and autonomic control (Mansour et al., 1994). MORs, in particular, are highly expressed at all levels of the central pain control network and mediate the canonical effects of clinically used opioids, namely pain relief (analgesia), euphoria, respiratory depression, and constipation. The most commonly prescribed opioid analgesics, including morphine, codeine, and hydrocodone, target the MOR. MOR stimulation within the descending pain modulatory system, which includes the PAG, rostral ventromedial medulla (RVM) and dorsal horn of the spinal cord (Basbaum and Fields, 1984), suppresses spinal cord nociceptive transmission and contributes to opioid-induced antinociception (Tortorici et al., 2001; Morgan et al., 2006;

Lueptow et al., 2018). Furthermore, dysregulation of μ-opioid signaling is strongly implicated in the transition to chronicity and the maintenance of chronic pain states (Schrepf et al., 2016; Thompson et al., 2018). As such, this thesis will largely focus on the μ-opioid system.

1.2 Mechanisms of pain and analgesia: A focus on the amygdala

The amygdala is a small nucleus within the medial temporal lobe that integrates sensory stimuli with affective valence. As part of the limbic system, the amygdala is involved in modulating emotional responses, memory, stress, and anxiety. Emerging evidence points to the amygdala as a key component of the pain matrix, responsible for mediating the emotional-affective elements of pain (Neugebauer et al., 2004; Bushnell et al., 2013; Jiang et al., 2014; Corder et al., 2019). The central nucleus of the amygdala (CeA) receives highly processed polymodal information from the thalamus and cortex, as well as nociceptive-specific inputs from the parabrachial nucleus (Gauriau and Bernard, 2002; Sah et al., 2003). The CeA has recently emerged as an important neural substrate for the bidirectional modulation of pain. In the following section, I will review the evidence describing: 1) how the CeA drives both pain amplification and suppression, and 2) how pain-related plasticity in this brain region promotes hypersensitivity in pathological states.

1.2-1 Cellular and circuit organization of the amygdala

The amygdala has been divided into several subregions based on genetic and circuit distinctions. It is comprised of two primary nuclei, the basolateral amygdala (BLA) and CeA, that are thought to signal in a serial fashion (Wassum and Izquierdo, 2015). Inputs to the BLA

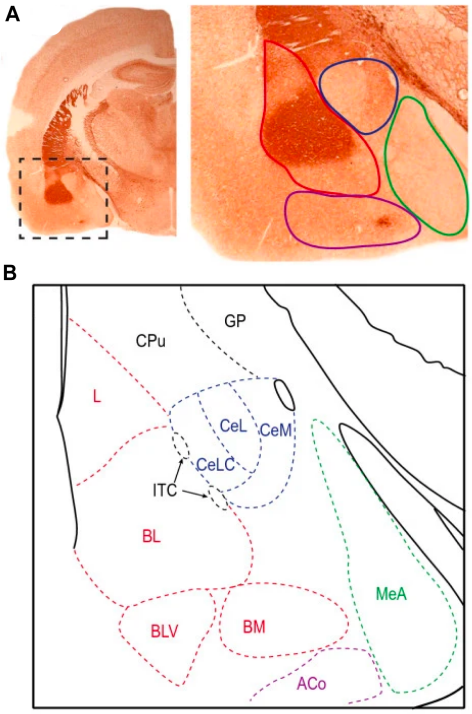


Figure 1-1. ***Anatomical organization of the amygdala.*** **A)** Representative image of the rodent brain at the level of the amygdala processed for acetylcholinesterase staining. The four main nuclei are outlined: central (blue), basolateral (red), medial (green), superficial (purple). **B)** Schematic representation of the amygdala nuclei. The central nucleus is represented with its capsular (CeLC), lateral (CeL) and medial (CeM) subdivisions. The basolateral group includes the lateral (L), basolateral (BL), basolateral ventral (BLV) and basomedial (BM) nuclei. CPu: caudate putamen; GP: globus pallidus; ITC, intercalated cell masses; MeA, medial nucleus; Aco; anterior cortical nucleus. Reprinted from Veinante P, Yalcin I, & Barrot M (2013). The amygdala between sensation and affect: a role in pain. *J Mol Psychiatr*, **1**, 9.

are largely from sensory, thalamic, and cortical regions, such as the insula, anterior cingulate cortex, and medial prefrontal cortex. Highly processed information generated in the BLA is relayed to the CeA, which serves as the major output nucleus for the amygdala and forms widespread connections with pain modulatory systems (Davis, 1998; LeDoux, 2000; Price, 2003; Veinante et al., 2013; Neugebauer, 2015). Within the CeA, the laterocapsular region (CeLC) is defined as the "nociceptive amygdala" because of its high content of neurons that process purely nociceptive information and respond preferentially to noxious stimuli (Bourgeais et al., 2001; Gauriau and Bernard, 2002; Neugebauer and Li, 2003). The CeLC receives direct nociceptive inputs from the spinal cord dorsal horn and parabrachial nucleus via the spino-parabrachio-amygdaloid tract, as well as affective and cognitive information from the BLA (Bernard and Besson, 1990; Burstein and Potrebic, 1993; Jasmin et al., 1997; Gauriau and Bernard, 2002; Veinante et al., 2013). The CeLC is therefore well-positioned to integrate nociceptive information with affective content, contributing to the

emotional-affective response to pain (Braz et al., 2005). Interposed between the BLA and CeA is an interconnected cluster of GABAergic neurons, termed the intercalated (ITC) cell mass. The ITC cells receive excitatory inputs from the medial prefrontal cortex and BLA, and gate amygdala output from the CeA through feedforward inhibition (Jüngling et al., 2008; Likhtik et al., 2008; Ren et al., 2013). Activation of ITC cells inhibits synaptic activation of CeLC neurons and attenuates pain-related behaviours (Ren et al., 2013). A general overview of pain-related amygdala neurocircuitry is illustrated in **FIG. 1-2.**

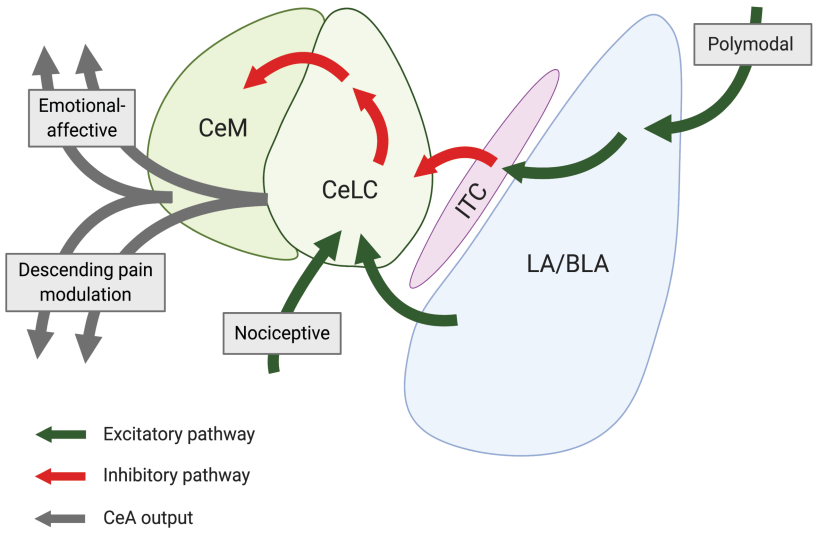


Figure 1-2. ***Amygdala pain-related neurocircuitry***. Input regions (lateral and basolateral amygdala, LA, BLA) receive and transmit polymodal sensory information from thalamocortical regions to the laterocapsular region of the central nucleus (CeLC) through direct excitatory projections or indirect feedforward inhibition involving interneurons in the intercalated (ITC) cell mass. The CeLC integrates nociceptive information received from the parabrachial nucleus via the spino-parabrachio-amygdaloid pathway with highly processed information from the LA-BLA network to produce emotional-affective responses and contribute to descending pain modulation via projections to the brainstem. CeM, medial subdivision of the central amygdala. Figure modeled after Thompson JM & Neugebauer V (2017). Amygdala plasticity and pain. *Pain research & management.*

The CeA contains functionally and neurochemically distinct populations of GABAergic neurons that primarily express either protein kinase C-δ (PKCδ) or somatostatin (SOM) (Li et al., 2013; Kim et al., 2017; Wilson et al., 2019). These two subpopulations of neurons receive excitatory nociceptive inputs from the parabrachial nucleus and modulate pain in an opposing manner via changes in excitability. In the context of nerve injury, cells expressing PKCδ are sensitized by nerve injury and amplify pain-related responses, whereas cells expressing SOM are inhibited by nerve injury and attenuate pain behaviours (Wilson et al., 2019).

1.2-2 The amygdala as a modulator of pain

Henry Molaison, known in the medical literature as Patient H.M., is one of the world's most famous amnesic patients. Patient H.M. underwent experimental neurosurgery in 1953, in which portions of the medial temporal lobe, including the amygdala, the uncus, and the hippocampus, were resected bilaterally to relieve medically intractable epilepsy. Following surgery, he developed profound anterograde amnesia (Scoville and Milner, 1957; Dossani et al., 2015). While modern scientific knowledge of how memory functions can be largely attributed to his case, re-evaluation of clinical reports in conjunction with post-mortem examination of his brain also suggest amygdala involvement in pain processing (Annese et al., 2014). Patient H.M. exhibited severe deficits in pain perception after the surgery, such that he did not perceive intense thermal noxious stimuli as painful when healthy controls did. His abnormal tolerance to pain is believed to be due to amygdala resection, highlighting the important role of the amygdala in pain modulation (Hebben et al., 1985; Dossani et al., 2015).

Numerous anatomical, behavioural, and physiological studies provide strong support for amygdala involvement in pain processes (Kulkarni et al., 2007; Neugebauer et al., 2009; Tillisch et al., 2011; Simons et al., 2014; Vachon-Presseau et al., 2016). Evidence from human neuroimaging studies indicate enhanced amygdala activation in healthy subjects in response to experimental noxious stimulation, including capsaicin application, mechanical compression, thermal stimulation, and a painful laser stimulus (Liu et al., 2010; Simons et al., 2014). Early electrophysiological *in vitro* and *in vivo* studies mapped amygdala neurons, located primarily in the CeLC, that respond preferentially to acute peripheral noxious stimuli (Bernard et al., 1992; Neugebauer and Li, 2002). Immunohistochemical markers of neuronal activity in the CeA are elevated in models of chronic neuropathic pain (Gonçalves and Dickenson, 2012), formalin-

induced inflammatory pain (Carrasquillo and Gereau IV, 2007, 2008), visceral pain (Traub et al., 1996; Crock et al., 2012), and acid-induced muscle pain (Cheng et al., 2011). Moreover, increasing CeA activity can generate or enhance pain responses even in the absence of tissue pathology (Carrasquillo and Gereau IV, 2007; Han et al., 2010; Kolber et al., 2010), whereas decreasing amygdala activity with lesions or pharmacological interventions reduces pain-related responses, such as vocalizations, shock-induced and thermal hyperalgesia, and post-stress analgesia in rodents (Werka and Marek, 1990; Crown et al., 2000; Han and Neugebauer, 2005; Ren et al., 2013). These data reinforce the pronociceptive role of the amygdala and suggest that changes in the activity and neurochemistry of the CeA in particular, contribute to the exacerbation of nociceptive responses.

1.2-3 The amygdala in descending modulation

In addition to its involvement in regulating pain perception, the amygdala has been established as an important neural substrate for opioid-dependent pain modulation, promoting pain reduction secondary to pharmacological interventions or stress. The amygdala regulates descending endogenous pain control through projections from the CeA to pain modulatory systems within diverse brain regions, including the PAG and RVM (Rizvi et al., 1991; Sun and Cassell, 1993; Pavlovic and Bodnar, 1998). MORs are highly expressed in the amygdala, particularly in GABAergic neurons of the CeA and the ITC masses, with fewer present in the BLA (Likhtik et al., 2008; Blaesse et al., 2015; Winters et al., 2017; Wang et al., 2018). The ITC cells are an interconnected cluster of GABAergic neurons found interposed between the BLA and CeA. MOR stimulation produces a net activation of the CeA through disinhibition of inhibitory projections from the ITC region, which in turn activates the descending pain

modulatory pathway (Da Costa Gomez and Behbehani, 1995; McGaraughty and Heinricher, 2002; Finnegan et al., 2005; Blaesse et al., 2015; Winters et al., 2017; Gregoriou et al., 2019). Early preclinical studies with rodent models highlight the pivotal contribution of MOR-mediated neurotransmission within the amygdala to endogenous and exogenous antinociception. For instance, lesion or inactivation of the CeA largely abolishes certain forms of morphine analgesia (Manning and Mayer, 1995; Manning, 1998) and stress-induced analgesia (Werka and Marek, 1990; Helmstetter, 1992; Helmstetter and Bellgowan, 1993; Fox and Sorenson, 1994). Direct microinjection of *β*-endorphin or morphine into the CeA attenuates nociceptive responses in the tail withdrawal test (Rodgers, 1978; Pavlovic et al., 1996). Additional evidence comes from *in vivo* electrophysiological recordings targeting nociceptive-specific neurons in the CeLC. Responses of CeLC neurons to noxious thermal and mechanical stimulation are suppressed by systemic morphine administration, providing further support for their role in antinociception (Huang et al., 1993). Finally, neuroimaging studies in healthy human subjects have shown that release of endogenous μ-opioid peptides in the amygdala during sustained pain largely correlates with reductions in pain intensity ratings (Zubieta et al., 2001, 2005; Bencherif et al., 2002). These results provide strong evidence that activation of the μ-opioid system in the amygdala is associated with an ongoing pain experience.

Importantly, a variety of chronic pain conditions have been linked with dysregulation of endogenous μ-opioid signaling within the amygdala. Human positron emission tomography (PET) studies show that compared with controls, patients with fibromyalgia, chronic back pain, rheumatoid arthritis, complex regional pain syndrome, and central neuropathic pain following stroke display reduced MOR availability at several loci along the neural axis, including the amygdala (Jones et al., 1994, 2004; Willoch et al., 2004; Harris et al., 2007; Klega et al., 2010;

Martikainen et al., 2013; Schrepf et al., 2016). Likewise, a recent study that employed PET and immunohistochemical techniques reported reduced MOR availability in the amygdala following nerve injury in rats (Thompson et al., 2018). Diminished opioid receptor availability may indicate increased occupancy by endogenous ligands or a reduction in the concentration or function of these receptors, thereby reflecting a fundamental remodeling in the brain that alters the capacity of those regions to respond to endogenous or exogenous opioids. In addition, chronic neuropathic pain induced by sciatic nerve ligation suppresses the ability of μ-opioid agonists to bind and activate MORs in membranes of the amygdala (Narita et al., 2006). Dysregulation of opioidergic signaling within the amygdala likely translates to inadequate descending antinociceptive activity and contributes to enhanced pain hypersensitivity in a wide range of chronic pain conditions.

1.2-4 Pain-related changes in amygdala neurocircuitry

Pain-related neuroplasticity in amygdala circuitry has been reported in a variety of rodent models of pain and linked with pain behaviours, suggesting that amygdala neuroplasticity is an important contributor to pathological pain states (Han and Neugebauer, 2004; Ikeda et al., 2007). Brain slice physiology studies have consistently shown enhanced synaptic transmission of excitatory nociceptive inputs from the parabrachial nucleus to the CeA, increased CeLC neuronal excitability, and irregular firing patterns in the CeLC in models of visceral pain (Han and Neugebauer, 2004), arthritis (Neugebauer et al., 2003; Bird et al., 2005; Fu and Neugebauer, 2008; Ren and Neugebauer, 2010), and neuropathic pain (Ikeda et al., 2007). CeLC neurons also display increased excitability and irregular firing patterns in models of formalin-induced inflammatory pain (Adedoyin et al., 2010). Rodents with spared nerve injury (SNI)-induced

chronic pain exhibit gross structural changes to the amygdala, including increased volumes of the BLA and CeA. This enlarged amygdala volume is associated with cell proliferation, perhaps as a result of the continuous input of nociceptive information into this region and the consequent prolonged and excessive stimulation (Gonçalves et al., 2008). The clinical relevance of alterations in amygdala neuroplasticity reported in preclinical animal pain models is corroborated by neuroimaging studies in humans. Converging PET and functional magnetic resonance imaging (fMRI) evidence indicate that amygdala activity is increased in humans with previously diagnosed pain conditions, including fibromyalgia, osteoarthritis, and irritable bowel syndrome compared to healthy controls (Kulkarni et al., 2007; Baliki et al., 2008; Tillisch et al., 2011; Simons et al., 2014). These findings support the notion that maladaptive neuroplastic changes in amygdala circuitry drive pain-related behaviours and dysregulated pain control.

1.3 Multiple sclerosis-related pain

Multiple sclerosis (MS) is a neuroinflammatory disease, characterized by chronic inflammation, demyelinated lesions, and neurodegenerative pathological processes within the CNS (Compston and Coles, 2008; Polman et al., 2011). MS is a highly prevalent chronic condition and a leading cause of disability in North America. Canada has one of the highest rates of MS in the world and an estimated 93,500 Canadians are currently living with the disease (Gilmour et al., 2018). While its etiology remains unknown, symptoms of MS result from damage to the myelin sheath and interruption of myelinated tracts in the CNS. In most patients, clinical manifestations of the disease include motor, cognitive, sensory, and autonomic disturbances, such as spasticity, loss of coordination and balance, deficits in executive functioning, vision impairment, and chronic pain (Compston and Coles, 2008). The clinical

course of MS may follow a variable pattern over time but can be classified into one of four categories: relapsing-remitting, secondary progressive, primary progressive, and progressive-relapsing (Lublin and Reingold, 1996). There is currently no cure for MS, although various forms of pharmaceutical and rehabilitation therapies are available to reduce MS attacks, slow disease progression, and manage symptoms (Gilmour et al., 2018).

Chronic pain is one of the most frequent and debilitating symptoms of MS, afflicting 50-80% of patients over the course of their disease (Österberg et al., 2005). MS-related pain is characterized by hyperalgesia (enhanced pain responses to noxious input) and allodynia (perception of innocuous/nonpainful stimuli as painful). In particular, MS patients most commonly describe the pain as constant, bilateral aching, burning, and pricking sensations in both the lower and upper extremities (Österberg et al., 2005). Classical pain treatments, such as opioid therapy, are typically ineffective in treating MS-related pain, with only a minority of patients receiving significant relief (Kalman et al., 2002). Despite the high prevalence and significant negative impact on quality of life, the underlying mechanisms of MS-related pain remain poorly understood.

1.3-1 Sex differences in pain and MS

For years, researchers and clinicians have noted that sexual disparities exist in a variety of chronic pain conditions (Berkley, 1997; Mogil and Bailey, 2010). However, the study of sex differences in pain in basic research settings has only recently become a topic of increased interest. The expansive body of literature in this field suggests that within patient populations, women are more likely to report the presence of pain, greater severity of pain, and longer duration of pain than men (Fillingim et al., 2009; Mogil, 2012). Differences in responsivity to

pharmacological and non-pharmacological pain interventions have also been noted (Keogh and Herdenfeldt, 2002; Keogh et al., 2005; Niesters et al., 2010). Further, highly prevalent chronic pain syndromes, including headache, neck pain, and osteoarthritis, have a marked female preponderance. This distinct female predominance extends to a variety of autoimmune diseases, including MS (Whitacre et al., 1999). The prevalence and incidence of MS in Canada is substantially higher in females compared to males, with ratios ranging from 2:1 to 3:1 (Österberg et al., 2005; Orton et al., 2006; Compston and Coles, 2008). Females with MS display greater levels of neuropathic pain than male patients (Moulin et al., 1988; Kalia and O'Connor, 2005). Epidemiologic and animal studies report that females have greater susceptibility to developing MS, while males often have worse disease progression (Golden and Voskuhl, 2017). Although the reasons for the increased incidence of pain and MS in females remain speculative, it has been proposed that a complex interaction between hormonal modulation, environment and epigenetic risk factors likely contribute to these differences (Voskuhl and Gold, 2012).

Recent advances in research using animal models have uncovered important biological differences in mediation of pain by the immune system (Totsch and Sorge, 2017). Our lab has previously demonstrated differences in spinal cord inflammation and plasticity between the sexes in the experimental autoimmune encephalomyelitis (EAE) model of MS, where EAE males with pain show greater neuronal plasticity and astrogliosis than females (Catuneanu et al., 2019). An increasing body of literature suggests that the influence of spinal microglia in pain initiation and maintenance is sex-specific (Sorge et al., 2015; Taves et al., 2015; Chen et al., 2018). SNI-induced chronic pain behaviour is proposed to be mediated by microglial-dependent pathways in males, whereas females preferentially use adaptive T-lymphocytes (Sorge et al., 2015; Taves et al., 2015). Intrathecal administration of microglial inhibitor, minocycline, suppresses SNI-,

formalin- and chronic constriction injury-induced pain behaviour exclusively in male mice, yet is effective in females with spinal cord injury and bone cancer pain (Chen et al., 2012; Yang et al., 2015). Although microglial signaling presents a clear sex dichotomy in pain, these results suggest that sex-dependent microglial signaling may not occur in all pain conditions or in all stages of pain development. It is evident that the biological processes underlying chronic pain are sexually distinct and may have significant impacts on disease prognosis and treatment outcomes. The consideration of sex in the study of MS-related pain pathophysiology is necessary to inform future efforts to develop sex-specific treatments.

1.3-2 Pain in EAE

EAE is an animal model most commonly used to study the pathophysiology of MS (Khan and Smith, 2014). EAE is a CD4+ T-lymphocyte-mediated demyelinating autoimmune disease of the CNS, characterized by widespread central inflammation and infiltration of T-cells and monocytes into the CNS (Robinson et al., 2014). Animals with EAE present many neuropathological features observed in MS patients, including neuroinflammation, demyelination, neurodegeneration, and axonopathy (Olechowski et al., 2009; Kipp et al., 2012; Potter et al., 2016; Catuneanu et al., 2019). In addition, affective disturbances (i.e. depression and anxiety-like behaviours) and cognitive and memory dysfunction have been noted early in the EAE disease course, similar to the clinical population (Pollak et al., 2002; Peruga et al., 2011; Acharjee et al., 2013; Olechowski et al., 2013).

Disease induction involves the active immunization of the animal, most commonly mice, with encephalitogenic antigens derived from CNS proteins, such as proteolipid protein peptide (PLP), myelin basic protein, and myelin oligodendrocyte glycoprotein (MOG), often emulsified

in an adjuvant such as Complete Freund's Adjuvant (CFA) (Kipp et al., 2012; Robinson et al., 2014). Peripheral immunization of susceptible mouse strains with these antigens results in the development of activated myelin-specific T-cells, immune cell trafficking into the CNS, and direct damage and inflammation in the CNS. Concomitant administration of pertussis toxin is commonly used to facilitate immune cell entry into the CNS through breakdown of the BBB. Notably, the combination of different rodent species and strain, immunization methods used and EAE inocula can generate different pain phenotypes. In the present study, mice were immunized with $MOG_{35–55}$ peptide, as this peptide is well-established to induce a chronic and progressive disease course in C57BL/6 mice (Rangachari and Kuchroo, 2013; Robinson et al., 2014).

Animals induced with $MOG_{35–55}$ EAE mirror many of the pronociceptive behaviours that are seen clinically in MS patients, such as mechanical allodynia, cold allodynia, and thermal hyperalgesia. The EAE model is therefore well-poised to study underlying pathophysiological mechanisms of pain in MS (Aicher et al., 2004; Olechowski et al., 2009; Rodrigues et al., 2009; Khan and Smith, 2014). As in MS, pain behaviours in EAE typically precede motor deficits (Österberg et al., 2005; Iannitti et al., 2014). Pain behaviour is often accompanied by robust CNS gliosis, glial activation, and inflammation throughout the course of disease (Olechowski et al., 2009; Rodrigues et al., 2009; Lu et al., 2012; Catuneanu et al., 2019). Pain-like behaviours in mice with EAE can be evaluated using a variety of standardized assays that assess sensory modalities, such as thermal and tactile sensitivity. Mice induced with MOG_{35-55} EAE develop significant mechanical hyperalgesia, as well as temperature and mechanical allodynia (Olechowski et al., 2009, 2013; Rodrigues et al., 2009; Yuan et al., 2012; Yousuf et al., 2017; Catuneanu et al., 2019). Although EAE is not an exact recapitulation of MS, it offers researchers a model to better understand the pathophysiological mechanisms involved in the MS clinical

disease course and the biological processes that drive MS-related pain. Use of the EAE model in rodents has been pivotal for the development of treatments to attenuate disability in MS and its continued application will provide important insight into the pathogenesis and management of chronic pain in MS.

1.3-3 MS and the amygdala

Adaptations within affective and motivational brain regions influence the experience of pain and analgesia, and disruptions to this circuitry contribute to pain chronicity (Elman et al., 2013; Taylor et al., 2015). MS is highly co-morbid with affective and mood disorders, suggesting that disturbances in affective circuitry are indeed prevalent within this patient population (Diaz-Olavarrieta et al., 1999; Korostil and Feinstein, 2007). Several studies have reported depression and anxiety-like symptoms in the EAE model (Pollak et al., 2002; Acharjee et al., 2018) and there is evidence indicating that amygdala function is affected early on in the MS disease course. Patients with MS display reduced amygdala volume and integrity, structural disorganization of the amygdala, and loss of functional connectivity with other cortical regions (Passamonti et al., 2009; Zhou et al., 2015; Wen et al., 2017; Hanken et al., 2018). These changes have been correlated with cognitive deficits and mood disorders associated with the disease (Pitteri et al., 2019). Despite the wealth of data implicating the amygdala as a critical node for pain modulation and analgesia (Simons et al., 2014; Thompson and Neugebauer, 2017), how alterations in amygdala function contribute to pain pathophysiology in MS and EAE remains unexplored. This knowledge is important to our mechanistic understanding of pain hypersensitivity and clinical pain reports in MS.

1.4 Aims and hypothesis

As outlined by the evidence provided above, chronic pain associated with MS represents a significant clinical and societal burden. As the general population ages, it can be expected that the rates of MS will only increase, and it is becoming increasingly imperative that adequate treatments for pain in this disease are developed. A more thorough understanding of the basic mechanisms driving this condition is necessary for the development of novel therapies to improve pain management for this patient population.

Therefore, the overall objective of this thesis was to investigate the relationship between amygdala activity, neuroinflammation, and endogenous pain control in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. I assessed nociceptive behaviour, morphine antinociception and morphine reward in male and female mice with EAE. I employed immunohistological methods to explore how inflammation within the central amygdala contributes to nociceptive processing and pain behaviours. Specifically, the three goals and hypothesis of the present study were as follows:

AIM 1: To evaluate the *in vivo* analgesic and reward function of MOR agonism in EAE.

AIM 2: To describe alterations in amygdala activity in response to pain and opioids in EAE.

AIM 3: To identify the mechanism of disrupted amygdala signaling in EAE.

I hypothesized that local inflammation within the central nucleus of the amygdala disrupts the balance of inhibitory and excitatory pain pathways that gate nociceptive and affective information in the EAE mouse model of MS.

CHAPTER 2: Materials and Methods

2.1 Animals and Ethics

All animal experiments and procedures were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with approval from the University of Alberta Health Sciences Animal Care and Use Committee. In total, male (N=96) and female (N=104) C57BL/6 mice aged 8-12 weeks-old were used in these experiments. Mice were housed in standard wire-top cages (4-5 per cage) in a temperature- and humidity-controlled environment with food and water available *ad libitum*. Mice were kept on a 12 h light/dark cycle and all experimental procedures were performed during light cycles only (9:00-17:00 hours). Prior to handling or behavioural testing, mice were allowed an acclimation period of 1 week upon arrival to the animal housing facility. A subsequent 3-4 days was spent handling the animals in the testing room to ensure familiarity of the experimenter and environment. Mice were distinguished from their cage mates with tail markings using non-toxic permanent markers.

2.2 EAE Induction

Male (N=49) and female (N=49) C57BL/6 mice (Charles River-Saint Constant Laboratory, QC, Canada) were used for the behavioural experiments. Following baseline testing periods, cages were randomly assigned to control (CFA) or experimental (EAE) groups. EAE was generated with $MOG_{35–55}$ peptide obtained from the Stanford University Peptide Synthesis Facility. To induce EAE, animals were immunized by subcutaneous injection of 50 µg/100 µL of $MOG_{35–55}$ emulsified in CFA (1.5 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) in the hind flank. All animals received an intraperitoneal (i.p.) injection of pertussis toxin (300 ng, List Biological Laboratories, Cedarlane, Canada), administered at the time of induction and 48 hours later in order to facilitate an immune response. The remaining mice received a

subcutaneous injection of CFA (1.5 mg/mL) and pertussis toxin only, without $MOG_{35–55}$ peptide, and were used as vehicle control for EAE induction.

2.3 Disease Scoring

EAE follows a predictable clinical course, characterized by a prodromal period of 10-18 days, followed by gradual ascending paralysis beginning in the tail and hind limbs and progressing to the forelimbs. Throughout all experiments, 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 (disease onset); Grade 2, mild hindlimb weakness with quick righting reflex; Grade 3, severe hindlimb weakness with slow righting reflex; Grade 4, hindlimb paralysis in one or both hindlimbs. No animals progressed past clinical Grade 1 and therefore did not exhibit any hindlimb weakness, paralysis, or righting reflex impairment.

2.4 Behavioural Testing

2.4.1 Thermal Tail Withdrawal Assay

The thermal tail withdrawal assay was used to measure baseline heat hypersensitivity and opioid-induced analgesia. Mice were gently restrained and approximately 2.5 cm of the tail was immersed in water warmed to 49°C. The latency to withdrawal (rapid flick of the tail) was measured until a maximum of 15 seconds to avoid tissue damage. A reduction in tail withdrawal latency (TWL) indicates hypersensitivity, whereas an increase suggests analgesia. Three baseline TWLs per animal were recorded and averaged. Data are expressed as either raw TWL or percent maximum possible effect (%MPE), defined as: [(test latency – baseline latency)/(cutoff latency – baseline latency)]*100.

A preliminary experiment was conducted to establish appropriate drug dosages. Naïve mice (N=16 males, N=16 females) received an injection of morphine (1 mg/kg or 10 mg/kg body weight, i.p., Western Drug Distribution Centre, Canada) dissolved in saline (0.9% NaCl) immediately following baseline measurements and underwent testing 30 minutes post-injection. A second cohort of male (N=20, which includes 10 CFA and 10 EAE) and female (N=20, which includes 10 CFA and 10 EAE) mice was used to test for thermal hyperalgesia during the pre-symptomatic stage of EAE. Baseline testing sessions were performed before EAE induction and experimental testing was performed prior to the onset of clinical motor symptoms (days 11-18 post-induction). A third cohort of animals (N=10 males, which includes 5 CFA and 5 EAE; N=10 females, which includes 5 CFA and 5 EAE) was used to assess morphine antinociception in the pre-symptomatic stage of EAE. Animals were injected with escalating doses of morphine sulfate (0.1-30 mg/kg, i.p.) and TWLs were measured every 20 minutes. Mice were returned to their home cages between testing.

2.4.2 Formalin Assay

The formalin assay was used to measure nociceptive behaviours and determine the effects of acute morphine administration in a model of subacute inflammatory/persistent pain. Prior to the start of the assay, each animal was placed into the testing apparatus for 10 minutes to habituate to the surroundings. The apparatus was a clear-walled plexiglass observation box (25 cm×23 cm×15 cm) with a raised platform to allow for an unobstructed view of the hind paw. A solution of 1% formalin was made by diluting 37% formaldehyde into 0.9% NaCl. Mice were lightly restrained and 30 µl of this solution was injected subcutaneously into the plantar surface of the left hindpaw. Each mouse was then placed back into the observation box and nociceptive

behaviours were monitored and timed for the first phase of the formalin response (0-10 min). The total time (in seconds) that mice spent licking/lifting/shaking was added together and reported as 'nociceptive response time'. Nociceptive behaviours were defined as licking or lifting of the injected paw, flinching, and vigorous shaking. Data are expressed as either raw nociceptive response time or percent analgesia (% analgesia), defined as: [mean nociceptive response time (control) – mean nociceptive response time (test)/mean nociceptive response time (control)]*100.

The analgesic effects of morphine on formalin-induced nociception in mice were first assessed in a cohort of naïve male mice (N=10). Mice received an intraperitoneal injection of saline vehicle (0.9% NaCl) or morphine sulfate (5 mg/kg) 20 minutes before testing. The mean nociceptive response time per animal was recorded. To assess morphine antinociception on formalin-evoked pain in EAE, male (N=19, which includes 9 CFA and 10 EAE) and female mice (N=19, which includes 10 CFA and 9 EAE) underwent testing as described above. Mice were tested on day of disease onset and did not exhibit any hindlimb weakness, paralysis, or righting reflex impairment. Animals that showed any signs of impairment that would interfere with the ability to exhibit nociceptive behaviours were excluded from analysis (N=2).

2.4.3 Conditioned Place Preference

Conditioned place preference (CPP) is a widely used model for studying the rewarding and aversive effects of drugs, such as morphine (Prus et al., 2009). The basic characteristics of this assay involve the association of a particular environment with drug treatment and the association of a different environment with the other drug (i.e. vehicle). An increase in the duration of time

spent in the drug-paired chamber versus the vehicle-paired chamber is associated with the rewarding properties of the drug and indicates a conditioned place preference.

This assay was conducted using a two-chamber, counter balanced and unbiased apparatus. Each chamber displayed distinct contextual characteristics. The CPP paradigm consists of three phases: habituation, conditioning, and testing. During habituation, the animals were allowed free access to both chambers for 30 minutes one day prior to conditioning. Baseline preference to the two chambers was assessed during the habituation session. After assessment of baseline preferences, half of the animals were assigned to receive 0.5 mg/kg morphine and the remaining half were assigned 10 mg/kg. Conditioning sessions consisted of mice receiving two trials of morphine sulfate (0.5 mg/kg or 10 mg/kg, i.p.) and two trials of saline (0.9% NaCl, i.p.) on alternating days over two days. Animals were confined to the chambers for 30 minutes following injection and immediately returned to their home cages. On the final testing day, mice were allowed free access to both chambers in a drug-free state and the time spent in the drug- and vehicle-paired chambers was assessed over 30 minutes.

To establish morphine dosages that reliably produce a place preference, we first conducted CPP testing with naïve male mice (N=16) using the protocol as described above. A second cohort of animals was used to assess morphine reward in EAE. Male (N=19, which includes 9 CFA and 10 EAE) and female mice (N=19, which includes 10 CFA and 9 EAE) were tested on days 5-8 post-disease induction. The distance travelled (cm) by each animal was acquired during testing and was used to ensure that locomotor function was retained for the duration of the experiment. Animals that showed signs of locomotor impairment or reached clinical Grade 1 during the testing period were excluded from analyses (N=1).

2.5 Stereotactic Lipopolysaccharide Injections

Male (N=15, which includes 7 VEH and 8 LPS) and female (N=39, which includes 19 VEH and 20 LPS) C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were anesthetized with isoflurane and mounted on a stereotaxic frame. Saline vehicle (0.9% NaCl, 100 nL per side) or lipopolysaccharide (LPS; 1 μg dissolved in 100 nL 0.9% NaCl per side; Sigma) was injected bilaterally into the CeA (coordinates from bregma: AP -1.06, ML ± 2.25, DV -4.50) using a sterile glass micropipette over a period of 1 minute per injection. Animals were allowed to recover for 72 hours following surgery, at which point thermal hypersensitivity (N=15 males; N=39 females) and morphine antinociception (N=15 males; N=15 females) were assessed in the tail withdrawal assay as previously described in *2.4.1 Thermal Tail Withdrawal Assay.* Data are expressed as either raw TWL or %MPE.

2.6 Tissue Collection and Preparation

On the day of disease onset (Grade 1), mice were euthanized using an intraperitoneal injection of Euthasol (sodium pentobarbital, 0.1 mL of 340 mg/mL). Animals that underwent testing in the formalin assay were euthanized 1-hour post-formalin treatment to allow for full expression of the cFOS protein. Animals were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The brain and lumbar spinal cords were dissected, post-fixed for 48 hours in 4% PFA at 4°C, and then transferred to 30% sucrose solution for 72 hours. Brains and spinal cords were embedded in Tissue Tek® O.C.T.™ (Optimal Cutting Temperature) compound (Fisher Scientific, Edmonton, AB, Canada), frozen on dry ice and stored at -80°C.

2.7 Immunohistochemical Analysis

Frozen brain and spinal cord tissue were cryosectioned (25 μm) and mounted onto glass slides. Tissue sections were washed 3 times for 10 minutes each in 1X phosphate-buffered saline (PBS) and blocked for 1 hour in PBS with 0.2% triton and 10% goat or donkey serum. Slides were then incubated at room temperature overnight in primary antibody solutions containing 1X PBS, 2% bovine serum albumin, 2% goat or donkey serum and appropriate antibody concentrations. The following primary antibodies and concentrations were used: rat anti-CD4 (1:200, Bio-Rad, USA), rabbit anti-Iba1 (1:500, Wako, USA), rabbit anti-cFOS (1:3000, Cell Signaling, USA), mouse anti-PKCδ (1:500, BD Biosciences, USA) and rat anti-somatostatin (1:250, Millipore, Canada). Following the incubation period, slides were washed as previously described before appropriate secondary antibodies were applied for 45 minutes in the dark at room temperature. Secondary immunolabeling was performed with the following antibodies and concentrations: donkey anti-rabbit Alexa Fluor 488 (1:200, Invitrogen-Life Technologies, Burlington ON, Canada), donkey anti-rat Alexa Fluor 594 (1:200), and donkey anti-mouse Alexa Fluor 647 (1:250). Tissue sections were counter stained and coverslipped using ProLong Gold mounting media containing fluorescent 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize cellular nuclei.

2.8 Fluorescent *in situ* Hybridization

Animals were euthanized either at disease onset or during the chronic stage of disease (day 8 post-onset) and the brain was promptly extracted and frozen on dry ice. Brain sections (12μm-thick) containing the amygdala were labelled with fluorescent *in situ* hybridization using RNAscope (Advanced Cell Diagnostics, Hayward, CA, USA) following the manufacturer's

protocol. A probe against *OPRM1* was used to label MOR mRNA in the CeA and ITC cell mass. To visualize cellular nuclei, tissue sections were counter stained with ProLong Gold mounting media containing DAPI (Invitrogen).

2.9 Image Acquisition and Analysis

All image acquisition and analyses were performed blind to experimental condition and sex. All measurements are the average of at least 3-5 images of both hemispheres of the brain or spinal cord per animal and numeric data was averaged within each animal during analysis. For each given immunohistochemical stain, only sections with identifiable nuclei were analyzed. Images for analysis were captured using a Zeiss AxioImager with either 10X, 20X or 63X objective lenses (Carl Zeiss, Germany). Representative images were acquired using a Zeiss AxioObserver inverted confocal microscope with 40X or 63X objective lenses (Carl Zeiss, Germany).

Iba-1, CD4, cFOS, SOM, and PKCδ stains were analyzed from 10X (Iba-1, CD4) or 20X (cFOS, SOM, PKCδ) magnification images using NIH ImageJ/FIJI software (a distribution of ImageJ). Iba-1 staining intensity (optical density) was assessed by measuring the area fraction of stain occupied within the region of interest. In order to objectively differentiate Iba-1-positive cells from diffuse staining, a staining intensity threshold that selectively identified concentrated staining surrounding the cell was applied to the images. The same threshold was applied to all images. Iba-1-positive microglial morphology (cell body size, process length, and branching) in the amygdala, NAc, and S1 was analyzed using MetaXpress® High Content Image Acquisition and Analysis Software from 63X magnification Z-stack confocal images. The morphology of microglial cells reflects their functional activation state (Zanier et al., 2015). Under normal,

physiological conditions, microglia present a ramified morphology. In response to pathological stimuli, microglia are readily activated and undergo a transformation to hypertrophied, ameboid morphology with retracted processes (Nimmerjahn et al., 2005).

The total number of CD4-, cFOS-, SOM-, and PKCδ-positive cells with DAPI-labeled nuclei were counted using the ImageJ Software Analyze Particles function with constant cell identification parameters across all images. Only clearly labeled and in-focus cells were counted in the entire optical region. For analysis of cFOS co-labelling with SOM- and PKCδ-positive cells, co-labelled cells were identified using ZenPro Image Processing (Zen Image Analysis) and manually counted. The number of *OPRM1*-positive cells in the CeA and ITC regions were quantified using ZenPro Image Processing (Zen Image Analysis) from 63X magnification images and calculated as a percentage of total cells within the region of interest.

2.10 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 9.0.0. In all experiments, data were tested for normality and parametric or non-parametric statistics were used accordingly. For comparison of two groups, unpaired two-tailed Student's t-test or Mann-Whitney test was performed. Two-way analysis of variance (ANOVA) or three-way ANOVAs with an appropriate post hoc analysis was used for comparisons of data sets with more than two groups or conditions. Significance threshold was set at $P < 0.05$ in all experiments. All data were expressed as mean ± standard error of the mean (SEM).

CHAPTER 3: Results

3.1 Establishing behavioural assays and experimental drug doses

3.1.1 Systemic morphine reduces thermal nociceptive hypersensitivity in naïve mice.

The thermal tail withdrawal assay, first described in 1941, is a commonly used test of thermal nociceptive thresholds (D'amour and Smith, 1941). We first sought to characterize the effects of acute morphine administration on nociceptive responses in naïve animals. To this end, we measured the thermal tail withdrawal thresholds of male and female animals at baseline and following morphine treatment. No significant difference in baseline TWL was noted between males and females (t-test, t=0.4374, ***p=0.6650***; **FIG. 3-1A**). Consistent with previous work (Lee et al., 2018), a single intraperitoneal dose of morphine (10 mg/kg) produced a significant effect on mean TWL in both sexes (two-way ANOVA, $F_{dose(2,57)}$=61.87**,** ***p<0.0001****,* $F_{sex(1,57)}$=1.412*,* ***p=0.2397****,* $F_{interaction(2,57)}$=4.699***, p=0.0129***; **FIG. 3-1B**).

3.1.2 Systemic morphine reduces formalin-induced nociceptive behaviours in naïve mice.

The formalin test is a widely accepted behavioural model of subacute chemogenic pain, which reliably induces pain behaviours such as licking and shaking of the injected paw (Coderre et al., 1993). Treatment with morphine has been shown to reduce formalin-induced nociceptive behaviours and produce analgesic effects in male and female rodents (Sevostianova et al., 2005; Gioiosa et al., 2008). To establish drug dosages for use in future experiments, we examined pain responses to intraplantar formalin in male mice pretreated with saline or morphine (5 mg/kg, i.p.). Animals treated with morphine displayed a significant reduction in the duration of nociceptive behaviours compared to saline-treated controls (t-test, t=4.381, ***p=0.0023***; **FIG. 3-1C**).

3.1.3 Systemic morphine induces a conditioned place preference in naïve mice.

We conducted CPP with naïve male mice to establish morphine dosages for use in future experiments. Previous work indicates that administration of 10 mg/kg morphine (i.p.) reliably induces a place preference in naïve animals, whereas doses of morphine below 1 mg/kg do not (Taylor et al., 2015). As expected, conditioning with 10 mg/kg morphine produced a significant place preference (t-test, t=3.830, ***p=0.0018***; **FIG. 3-1D**). No conditioned place preference was found when animals were administered 0.5 mg/kg morphine (t-test, t=1.087, ***p=0.2954***; **FIG. 3-1E**).

3.2 EAE as a model of MS-related pain

3.2.1 Pre-symptomatic EAE is associated with thermal hypersensitivity.

In humans, patients often present with pain as an initial sign of MS or as their only symptom prior to the development of other symptoms (Marchettini et al., 2006). Robust tactile and cold allodynia have been well-documented in the pre-symptomatic stages of EAE (Olechowski et al., 2009). To determine whether the early stages of disease are also associated with heat hyperalgesia, we employed the thermal tail withdrawal assay prior to the onset of clinical motor symptoms (**FIG. 3-2A**). We found that in the pre-symptomatic stage of disease (days 11-18), male and female mice with EAE displayed significantly lower thermal TWLs than CFA-treated controls (two-way ANOVA, $F_{disease(1,56)}$=10.35, ***p=0.0022***, $F_{time(1,56)}$=8.826, ***p=0.0044***, $F_{interaction(1,56)}$=5.558, ***p=0.0219***; **FIG. 3-2B**), suggesting that these animals were experiencing heat hyperalgesia (enhanced sensitivity to noxious heat). No significant sex differences were detected; therefore, males and females were pooled for these analyses (three-way ANOVA, $F_{sex(1,52)}$=1.636**,** ***p=0.2066***; **Supplementary FIG. 3-1**).

3.2.2 Pre-symptomatic EAE is associated with microgliosis and T-cell infiltration in the spinal cord.

Markers of inflammation and microgliosis were used to assess whether animals with EAE display disease pathologies prior to the onset of clinical motor symptoms. This pre-symptomatic timepoint was associated with cellular signs of disease, including microgliosis and T-cell infiltration in the lumbar spinal cord (**FIG. 3-2C-F**). The number of Iba-1+ microglial cells (t-test, t=2.277, ***p=0.0488***; **FIG. 3-2E**) and CD4+ T-cells (t-test, t=2.650, ***p=0.0243***; **FIG. 3-2F**) in the lumbar spinal cord were significantly increased in EAE mice compared to CFA-treated controls. These results indicate that EAE is associated with changes in pain sensitivity, as well as microglial and immune cell reactivity within the spinal cord that precede deficits in locomotor function.

3.3 MOR-mediated behavioural responses in EAE

3.3.1 Antinociceptive effects of morphine on thermal pain sensitivity in EAE are sex-dependent.

Morphine-induced analgesia in EAE was measured with the tail withdrawal assay. Escalating doses of systemic morphine (0.1-30 mg/kg, i.p) produced dose-related elevations in withdrawal thresholds (analgesia) that were not significantly different between male EAE and CFA animals (two-way ANOVA, $F_{disease(4,40)}$=3.333, ***p=0.0754***, $F_{dose(4,40)}$=155.1, ***p<0.0001***, $F_{interaction(4,40)}$=0.7390, ***p=0.5719***; **FIG. 3-3A**). Systemic morphine produced a large dose-dependent antinociceptive response on TWL in female CFA controls; however, this analgesic effect was significantly reduced in female mice with EAE (two-way ANOVA, $F_{disease(1,40)}$=29.29, ***p<0.0001***, $F_{dose(4,40)}$=177.5, ***p<0.0001***, $F_{interaction(4,40)}$=9.261, ***p<0.0001***; **FIG. 3-3A**). Significant

sex differences in morphine antinociception were noted, such that EAE males displayed significantly greater TWLs than females (three-way ANOVA, $F_{sex(1,80)}$=66.0, ***p<0.0001***). This sexually dimorphic effect was most evident at higher doses of morphine (Sidak's *post-hoc* comparison, 10 mg/kg, ***p=0.0048***; 30 mg/kg, ***p<0.0001***). These comparisons remained when data were expressed as %MPE induced by 30 mg/kg morphine (two-way ANOVA, $F_{disease(1,16)}$=13.21, ***p=0.0022***, $F_{sex(1,16)}$=31.63, ***p<0.0001***, $F_{interaction(1,16)}$=11.48, ***p=0.0037***; **FIG. 3-3B**). These data suggest that MOR-mediated antinociception is sexually dimorphic in EAE.

3.3.2 Antinociceptive effects of morphine on formalin-induced pain behaviour in EAE are sex-dependent.

We next sought to examine the antinociceptive effects of systemic morphine on pain behaviours elicited by persistent noxious stimulation in EAE. We therefore assessed nociceptive behaviour in response to intraplanar formalin in male and female EAE mice pretreated with either saline vehicle or morphine (5 mg/kg, i.p.). As previously reported by Olechowski et al. (2010), pain behaviours in the formalin assay were dramatically reduced in mice with EAE compared to CFA-treated controls (**FIG. 3-3C**). Morphine pretreatment induced significant analgesia and attenuated the duration of nociceptive behaviours in CFA controls. In contrast, morphine had no significant antinociceptive effect on formalin-evoked pain behaviours in male (two-way ANOVA, $F_{disease(1,15)}$=7.232, ***p=0.0168***, $F_{drug(1,15)}$=10.35, ***p=0.0058***, $F_{interaction(1,15)}$=7.119, ***p=0.0175***; **FIG. 3-3C**) or female mice with EAE (two-way ANOVA, $F_{disease(1,13)}$=35.75, ***p<0.0001***, $F_{drug(1,13)}$=42.10, ***p<0.0001***, $F_{interaction(1,13)}$=89.77, ***p<0.0001***; **FIG. 3-3C**). Notably, this loss of morphine antinociception was significantly more pronounced in EAE females than males (three-way ANOVA, $F_{sex(1,28)}$=6.647, ***p=0.0155***). These comparisons

remained when data were expressed as % analgesia (two-way ANOVA, $F_{disease(1,14)}$=117.9***, p<0.0001***, $F_{sex(1,14)}$=10.98***, p=0.0051,*** $F_{interaction(1,14)}$=8.871***, p=0.0100***; **FIG. 3-3D**).

3.3.3 Morphine reward is impaired in EAE.

We have previously shown that chronic pain causes disruptions within affective and reward circuitry, resulting in impaired reward behaviour (Taylor et al., 2015). To explore how EAE influences the rewarding properties of morphine, we used the CPP paradigm to test for preference to morphine in mice with or without EAE during the pre-symptomatic stage of disease (days 5-8). As no significant sex differences were detected when animals received 10 mg/kg morphine (three-way ANOVA, $F_{sex(1,28)}$=0.01428, ***p=0.9057***; **Supplementary FIG. 3-2A**), or 0.5 mg/kg (three-way ANOVA, $F_{sex(1,30)}$=0.08526, ***p=0.7723***; **Supplementary FIG. 3-2B**), males and females were pooled for these analyses. There was a significant effect of drug-pairing when animals were administered 10 mg/kg morphine (two-way ANOVA, $F_{drug-pairing(1,32)}$=11.88, ***p=0.0016***, $F_{disease(1,32)}$=0.0.006437, ***p=0.9366***; **FIG. 3-4A**). We noted a significant interaction between drug-pairing and disease ($F_{interaction(1,32)}$=5.132, ***p=0.0304***), where morphine induced a place preference in CFA-treated controls (Sidak's *post-hoc* comparison, ***p=0.0003***), but not EAE animals (Sidak's *post-hoc* comparison, ***p=0.6793***). Conditioning with 0.5 mg/kg morphine produced a significant effect of drug-pairing (two-way ANOVA, $F_{drug-pairing(1,34)}$=7.405, ***p=0.0102***, $F_{disease(1,34)}$=0.009987, ***p=0.9210***; **FIG. 3-4B**) and an interaction between drug-pairing and disease ($F_{interaction(1,34)}$=4.991, ***p=0.0322***). In particular, this low dose of morphine did not produce a place preference in CFA controls (Sidak's *post-hoc* comparison, ***p=0.9319***), and intriguingly, elicited a place aversion in EAE animals (Sidak's *post-hoc*

comparison, ***p=0.0020***). Collectively, these results indicate a loss of reward-like behaviour in EAE.

To confirm that mice with EAE had no locomotor deficits that would interfere with the ability to develop or exhibit a place preference, locomotor activity in the chamber was recorded during conditioning sessions and on the final test day in a drug-free state (**FIG. 3-4C**). No significant deficits in baseline locomotor function (t-test, t=1.075, ***p=0.2993***) or in morphine-induced hyperlocomotion (t-test, t=0.5431, ***p=0.5937***) were observed at this stage of disease.

3.4 Microglial activation in sensory and affective brain regions in EAE

3.4.1 EAE is associated with microglial activation in the amygdala.

Mounting evidence suggests that inflammation and changes in microglial cell reactivity can influence pain sensitivity in a variety of injury and pain models (Svensson et al., 2003; Coull et al., 2005; Tsuda et al., 2009; Beggs et al., 2012; Sorge et al., 2015; Taylor et al., 2015; Barcelon et al., 2019). While reactive gliosis and inflammation at the spinal level are established characteristics of the EAE model, few studies have examined the contribution of microglia in the brain to EAE-induced pain. Sensory deficits and mood disorders are highly prevalent among MS patients (Schubert and Foliart, 1993; Diaz-Olavarrieta et al., 1999), thus we explored a possible role for microglia in sensory and affective regions in EAE. On the basis of Iba-1 immunolabeling, EAE animals showed significant microglial activation in the amygdala, but not in the primary somatosensory cortex or NAc, at the onset of disease (**FIG. 3-5A**). At this stage of disease, Iba-1+ amygdala microglia exhibited a stereotypical activated phenotype, including enlarged cell body size (t-test, t=3.221, ***p=0.0034***; **FIG. 3-5B**), reduced cell branching (t-test, t=3.353, ***p=0.0025***; **FIG. 3-5C**), and shorter mean process length (t-test, t=5.715, ***p<0.0001***;

FIG. 3-5D). We have previously shown that these morphological changes correlate with the activation state of microglia (Taylor et al., 2017). Moreover, we noted a significant increase in Iba-1+ immunostaining specifically in the CeA of EAE animals compared to CFA controls (t-test, t=2.168, ***p=0.0430***; **FIG. 3-5E**). We did not detect any significant sex differences in microglial cell body size in the amygdala (two-way ANOVA, $F_{sex(1,24)}$=2.156, ***p=0.1550***), somatosensory cortex ($F_{sex(1,27)}$=1.447, ***p=0.2395***) or NAc ($F_{sex(1,27)}$=1.268, ***p=0.2701***; **Supplementary FIG. 3-3A**). Similarly, no sex differences in microglial cell branching ($F_{sex(1,24)}$=1.774, ***p=0.1954***; **Supplementary FIG. 3-3B**), process length ($F_{sex(1,24)}$=0.01068, ***p=0.9185***; **Supplementary FIG. 3-3C**) or staining density ($F_{sex(1,17)}$=0.07796, ***p=0.7835***; **Supplementary FIG. 3-3D**) were detected. As such, the sexes were pooled for these analyses. It may be noted that Iba-1 stains both microglia and macrophages, so we must consider that some labeled cells may be peripheral macrophages that have infiltrated the brain.

3.5 Nociceptive signaling in EAE

3.5.1 Nociceptive signaling within the CeA is altered in EAE.

The CeA receives direct nociceptive projections from the spinal cord and parabrachial nucleus via the spino-parabrachio-amygdaloid pathway, playing a critical role in nociceptive processing (Bernard and Besson, 1990; Burstein and Potrebic, 1993; Gauriau and Bernard, 2002). Recent work indicates that the CeA contains two populations of GABAergic neurons (PKCδ+ and SOM+) that regulate pain perception in an opposing manner (Wilson et al., 2019). PKCδ+ neurons are activated by nociceptive stimuli and increase pain behaviours, whereas activation of SOM+ neurons drives antinociception. This work also found that nociceptive activity within the CeA is altered in chronic neuropathic pain. Here, we investigated how CeA

activity is altered in EAE using cFOS immunolabeling. cFOS is a nuclear transcription factor that is commonly used as an immunohistochemical marker for neuronal activation (Gao and Ji, 2009). No significant sex differences were found in total cFOS+ cell counts (three-way ANOVA, $F_{sex(1,40)}$=0.07902, ***p=0.7801***; **Supplementary FIG. 3-4A**), cFOS co-labelling with SOM+ ($F_{sex(1,36)}$=0.6429, ***p=0.4279***; **Supplementary FIG. 3-4B**) or PKCδ+ cells ($F_{sex(1,38)}$=1.560, ***p=0.2192***; **Supplementary FIG. 3-4C**) in either control or EAE animals; therefore, males and females were pooled for these analyses.

On day of disease onset, EAE animals displayed significantly more CeA-cFOS+ cells compared to CFA controls (t-test, t=2.255, ***p=0.0366***; **FIG. 3-6B**). To determine the neuronal identity of the cFOS+ cells in the EAE tissue, we co-labelled for cFOS alongside markers for SOM (**FIG. 3-6A**) and PKCδ (**FIG. 3-7A**). We found no significant differences in the total number of SOM+ (t-test, t=0.3505, ***p=0.7290***; **FIG. 3-6C**) or PKCδ+ neurons (t-test, t=0.1145, ***p=0.9098***; **FIG. 3-7B**) between CFA and EAE mice. Systemic morphine evoked a dramatic increase in the activity of antinociceptive CeA-SOM+ cells in CFA mice, but failed to elicit this response in EAE animals (two-way ANOVA, $F_{drug(1,41)}$=33.43, ***p<0.0001***, $F_{disease(1,41)}$=7.632, ***p=0.0085***, $F_{interaction(1,41)}$=14.63, ***p=0.0004***; **FIG. 3-6D**). We hypothesized that EAE would promote activity in pronociceptive (PKCδ+) neurons, thus contributing to pain hypersensitivity in the disease. cFOS co-labelling with PKCδ was not found to be significantly different between CFA and EAE animals; however, the data indicates a trend towards increased PKCδ activity in EAE (two-way ANOVA, $F_{disease(1,41)}$=3.622, ***p=0.0641***; **FIG. 3-7C**). Morphine treatment had no significant effect on cFOS/PKCδ colocalization in either controls or EAE mice ($F_{drug(1,41)}$=2.177, ***p=0.1478***; $F_{interaction(1,41)}$=0.06284, ***p=0.8033***). Taken together, these results indicate that a loss of

responsivity within the antinociceptive neurons in the CeA might be driving impaired morphine efficacy in EAE.

3.5.2 EAE has no significant effect on formalin-evoked cFOS immunoreactivity in the CeA.

Noxious stimulation of the hindpaw with intraplantar formalin increases the number of cells expressing cFOS in the CeA (Lu et al., 2014; Miyazawa et al., 2018). In contrast, systemic opioid administration reduces pain-evoked cFOS expression in the spinal cord and supraspinal sites involved in pain modulation, such as the parabrachial nucleus (Jasmin et al., 1994; Ebersberger et al., 1995; Sawamura et al., 1999; Nazarian et al., 2008). We observed that morphine analgesia to intraplantar formalin injection was dramatically diminished in EAE mice (**FIG. 3-3**). Therefore, we sought to investigate how these behavioural responses correlated with changes in CeA neuronal activity. To examine formalin-evoked cellular activation within the nociceptive circuitry of the CeA, we stained for cFOS 1-hour post-formalin exposure in CFA and EAE mice pretreated with either saline or morphine (**FIG. 3-8A**). Overall, CeA-FOS expression did not differ between the sexes (three-way ANOVA, $F_{sex(1,27)}$=0.04324, ***p=0.8368***; **Supplementary FIG. 3-5**). Morphine pretreatment led to a reduction in formalin-evoked cFOS expression in the CeA, which was not significantly different between CFA and EAE animals (two-way ANOVA, $F_{drug(1,31)}$=5.246, ***p=0.0290***, $F_{disease(1,31)}$=1.826, ***p=0.1864***, $F_{interaction(1,31)}$=5.815e-007, ***p=0.9994***; **FIG. 3-8B**). These results indicate that the observed lack of morphine antinociceptive potency on formalin-induced pain in EAE **(FIG. 3-3)** is not due to alterations in signaling at the level of the CeA. Rather, these findings suggest inherent mechanistic differences in morphine action in the absence versus the presence of persistent inflammatory pain in EAE.

3.5.3 EAE has no significant effect on OPRM1 expression in the CeA or ITC cell mass.

μ -opioid agonists, such as morphine, produce their analgesic effects through binding to the MOR (encoded by *OPRM1* gene). We hypothesized that alterations in *OPRM1* expression within the CeA and surrounding regions might be associated with EAE-induced chronic pain, as well as correlate with the loss of opioid-induced analgesia and impaired reward in EAE animals. Using fluorescent *in situ* hybridization, we evaluated *OPRM1* expression in the CeA and ITC cell mass at EAE onset and in the chronic phase of disease (**FIG. 3-9**). We found no significant differences in the number of *OPRM*1+ cells in the CeA between CFA control and EAE animals at the onset of disease (t-test, t=0.8018, ***p=0.4490***; **FIG. 3-9B**) or during chronic EAE (t-test, t=2.003, ***p=0.1015***; **FIG. 3-9B**). Total CeA-*OPRM1* expression was not significantly different between CFA-treated controls and animals with chronic EAE (t-test, t=1.102, ***p=0.2989***; **FIG. 3-9B**). Similarly, we found no significant differences in the number of *OPRM1*+ cells in the ITC region between CFA and EAE animals at disease onset (t-test, t=0.7206, ***p=0.4945***; **FIG. 3-9C**) or in the chronic phase (t-test, t=0.7987, ***p=0.4450***; **FIG. 3-9C**). The sample size for this experiment was not large enough to evaluate the effect of sex on *OPRM1* expression.

3.6 Microglial activation in the central amygdala

3.6.1 Intra-CeA injection of LPS induces focal microglial activation.

Microglial activation throughout the neural axis plays a central role in the development and maintenance of chronic pain (Beggs et al., 2012; Taylor et al., 2017). Given the robust microglial activation observed specifically within the amygdala (**FIG. 3-5**), we next asked whether activated microglia contribute to the altered central amygdala nociceptive processing in EAE. Naïve male and female mice received bilateral stereotaxic microinjections of saline vehicle or

LPS into the CeA to focally activate microglia (**FIG. 3-10A**). The ability of LPS to activate microglia in the murine brain after stereotaxic injection is well-established (Castaño et al., 1998; Arai et al., 2004). We confirmed that intra-CeA LPS induced significant microglial activation in both sexes, as evidenced by increased cell body size of Iba-1 immunopositive cells surrounding the injection site (two-way ANOVA, $F_{treatment(1,27)}$=165.6, ***p<0.0001***, $F_{sex(1,27)}$=2.775, ***p=0.1073***, $F_{interaction(1,27)}$=2.277, ***p=0.1430***; **FIG. 3-10D**). Intra-CeA injection of LPS did not induce microglial activation in surrounding areas such as the secondary sensory cortex, indicating that LPS treatment did not lead to diffuse inflammation throughout the brain ($F_{treatment(1,26)}$=3.260, ***p=0.0826***, $F_{sex(1,26)}$=0.4877, ***p=0.4912***, $F_{interaction(1,26)}$=0.1284, p=0.7229; **FIG. 3-10C**). Although we confirmed that microglial activation was limited to the region surrounding the injection site, we acknowledge that LPS-induced inflammation likely extended throughout the amygdala and was not strictly limited to the CeA.

3.6.2 Activated microglia in the CeA attenuate MOR-mediated analgesia in female mice.

Seventy-two hours following intra-CeA injection of LPS, thermal pain hypersensitivity and morphine analgesia were measured with the tail withdrawal assay. Baseline TWLs were not significantly different between male and female mice treated with saline vehicle or LPS (two-way ANOVA, $F_{treatment(1,50)}$=2.878, ***p=0.0960***, $F_{sex(1,50)}$=1.792, ***p=0.1868***, $F_{interaction(1,50)}$=0.0003599, ***p=0.9849***; **FIG. 3-11A**). Morphine produced dose-dependent increases in withdrawal thresholds that were not significantly different between saline- and LPS-treated males (two-way ANOVA, $F_{treatment(4,65)}$=1.179, ***p=0.2815***, $F_{MOR\ dose(4,65)}$=143.0, ***p<0.0001***, $F_{interaction(4,65)}$=0.8252***, p=0.5139***; **FIG. 3-11B**). Strikingly, injection of LPS into the CeA interfered with morphine antinociception in females, such that the degree of analgesia produced

by morphine was significantly greater in females treated with saline than with LPS (two-way ANOVA, $F_{treatment(1,65)}$=7.591, ***p=0.0076***, $F_{MOR\ dose(4,65)}$=145.3, ***p<0.0001***, $F_{interaction(4,65)}$=3.003, ***p=0.0245***; **FIG. 3-11B**). As shown in Figure 3-11, significant sex differences in the antinociceptive potency of morphine were detected. In comparison to males, morphine analgesia was significantly attenuated in LPS-treated females (three-way ANOVA, $F_{sex(1,26)}$=9.578, ***p=0.0047***). These trends remained when data were transformed to %MPE induced by 30 mg/kg morphine (two-way ANOVA, $F_{sex(1,25)}$=3.947, ***p=0.0580***, $F_{treatment(1,25)}$=3.017, ***p=0.0947***, $F_{interaction(1,25)}$=6.889, ***p=0.0146***; **FIG. 3-11C**). These findings indicate that the contribution of microglial activation in the CeA to MOR-mediated antinociception is sex-dependent.

3.7 Figures

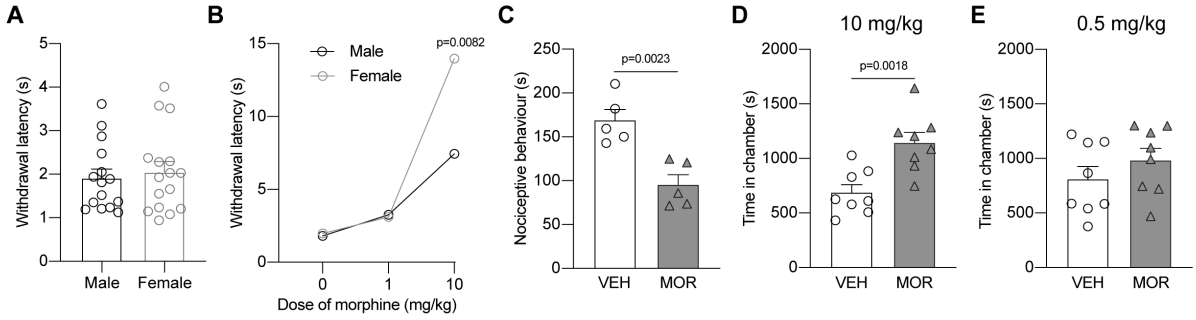


Figure 3-1. ***Systemic morphine produces antinociception and reward in naïve mice.*** **(A)** Baseline thermal tail withdrawal latencies did not differ between male and female mice. **(B)** Morphine dose-response was measured using thermal tail withdrawal and compared between male and female animals. **(C)** Acute morphine treatment (5 mg/kg) attenuated the mean duration of nociceptive behaviour evoked by intraplantar formalin. **(D, E)** Conditioned place preference to morphine (10 mg/kg and 0.5 mg/kg) in naïve male mice. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. VEH, Vehicle; MOR, morphine.

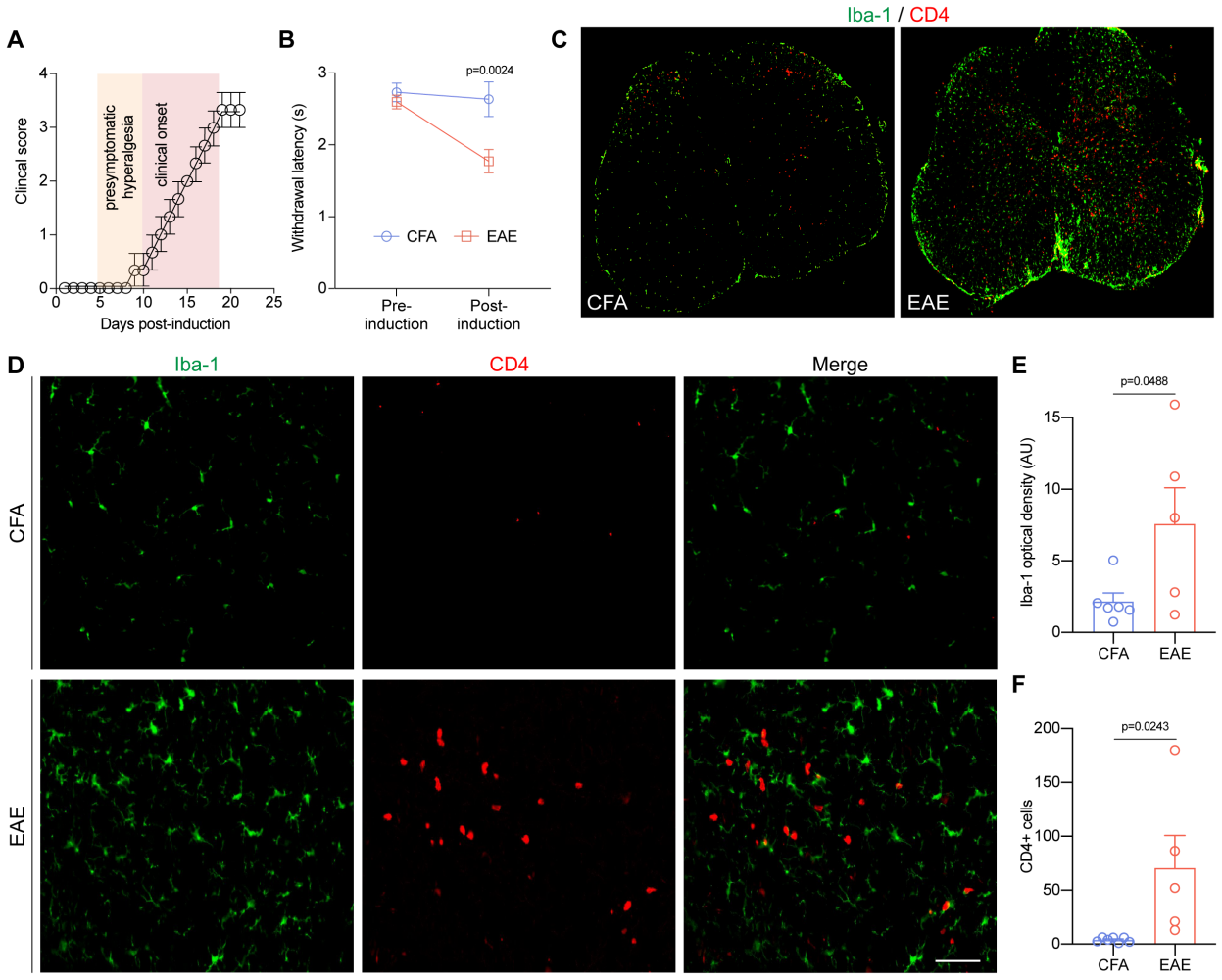


Figure 3-2. ***EAE as a model of MS-related pain.*** **(A)** Schematic of typical EAE disease course and progression. **(B)** Baseline thermal tail withdrawal thresholds compared between CFA controls and EAE animals before and after disease induction. **(C)** Representative images of the lumbar spinal cord stained with Iba-1 (microglia, **green**) and CD4 (CD4+ T cells, **red**). **(D)** Representative 20X photomicrographs of Iba-1 and CD4 immunostaining in the spinal cord of CFA control and EAE animals. Scale bar = 100 μm, applies to all images. The number of infiltrating **(E)** Iba+ and **(F)** CD4+ cells in the lumbar spinal cord were significantly increased in the pre-symptomatic stage of EAE. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. AU, arbitrary units; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

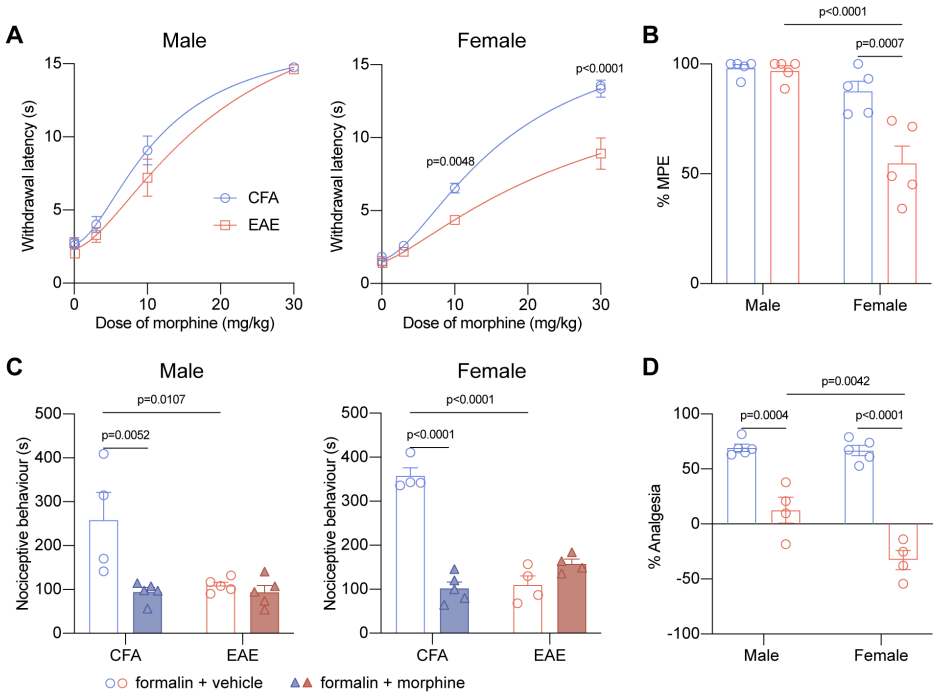


Figure 3-3. ***Morphine-induced analgesia is diminished in female mice with EAE.*** **(A)** Morphine (0.1-30 mg/kg) dose-response curve was measured using thermal tail withdrawal and compared between male and female CFA control and EAE animals. Morphine antinociception was significantly reduced in female mice with EAE. Pairwise post-hoc analyses by Sidak's multiple comparisons test. **(B)** Transformed data (%MPE = percent maximum possible effect) of tail withdrawal thresholds from animals treated with 30 mg/kg morphine. Pairwise post-hoc analyses by Tukey's multiple comparisons test. **(C)** Systemic morphine (5 mg/kg) attenuated formalin-evoked nociceptive behaviour in male and female CFA controls and had no significant effect in EAE animals. **(D)** Transformed data (% analgesia) of mean nociceptive response duration from animals treated with morphine (5 mg/kg). Pairwise post-hoc analyses by Tukey's multiple comparisons test. Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

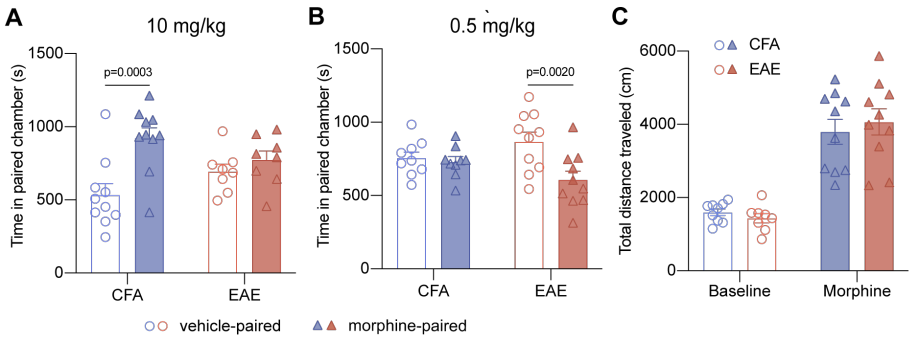


Figure 3-4. ***Morphine-induced conditioned place preference is impaired in EAE.*** Conditioned place preference to systemic morphine compared between CFA-treated controls and EAE mice. **(A)** Control animals spent significantly more time in the morphine (10 mg/kg)-paired chamber. Morphine failed to elicit a conditioned place preference in mice with EAE. **(B)** Low-dose morphine (0.5 mg/kg) did not induce a place preference in CFA-treated animals and produced a place aversion in EAE animals. **(C)** Locomotor function was not affected in EAE mice at this stage of disease. Mice with EAE showed no impairments in basal locomotor activity, nor in morphine-induced hyperlocomotion when compared with controls. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

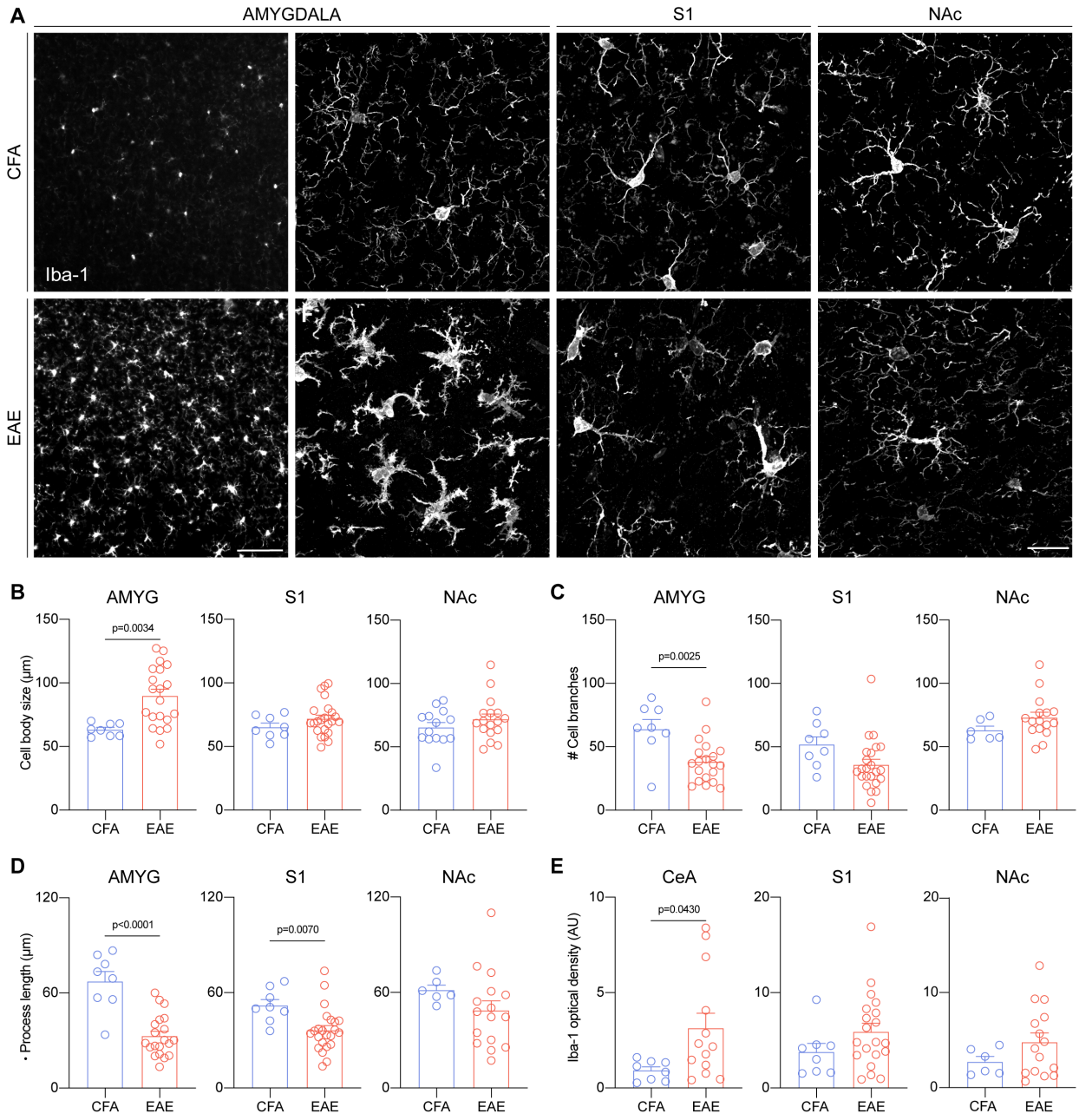


Figure 3-5. ***EAE induces microglial activation in the amygdala, but not in the S1 or NAc.*** **(A)** Representative photomicrographs of Iba-1 immunostaining in the amygdala, primary somatosensory cortex (S1), and nucleus accumbens (NAc) of CFA control and EAE animals. Scale bar in bottom left panel = 100 µm, also applies to top left panel. Scale bar in bottom right panel = 20 µm, applies to all other images. Quantification of **(B)** microglial cell body size, **(C)** number of cell branches, and **(D)** mean process length in select brain regions of CFA and EAE mice. Amygdala microglia displayed an activated phenotype at the onset of EAE. There was no significant effect in the S1 or NAc. **(E)** Iba-1+ staining density was significantly increased in the central amygdala of EAE mice compared to controls. Data presented as mean ± SEM. AMYG, amygdala; AU, arbitrary units; CeA, central nucleus of the amygdala; CFA, Complete Freund's

Adjuvant; EAE, experimental autoimmune encephalomyelitis; NAc, nucleus accumbens; S1, primary somatosensory cortex.

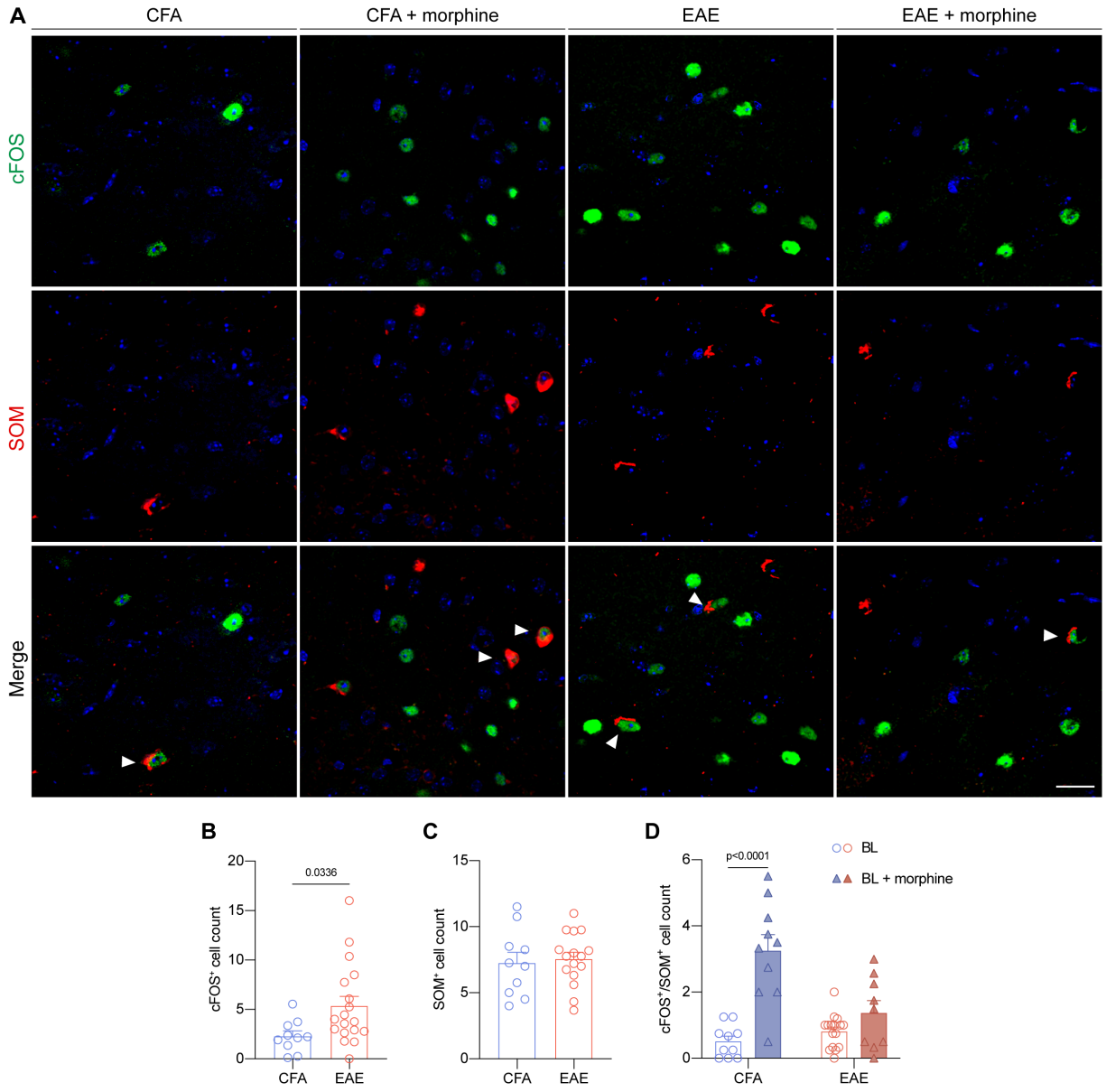


Figure 3-6. ***Antinociceptive signaling in the CeA is dysregulated in EAE.*** **(A)** Representative 40X photomicrographs of cFOS **(green)** and SOM **(red)** immunostaining in the central amygdala of CFA and EAE animals with and without an acute morphine (5 mg/kg, i.p.) challenge. White arrowheads indicate co-labelled cFOS+/SOM+ neurons. Scale bar = 20 µm, applies to all images. **(B)** The total number of CeA-cFOS+ cells was significantly increased in EAE animals compared to CFA controls. **(C)** The total number of CeA-SOM+ cells was not significantly different between CFA and EAE animals. **(D)** Systemic morphine induced a significant increase in cFOS co-labelling with SOM+ cells in the central amygdala of CFA controls, but not EAE mice. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. BL, baseline; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

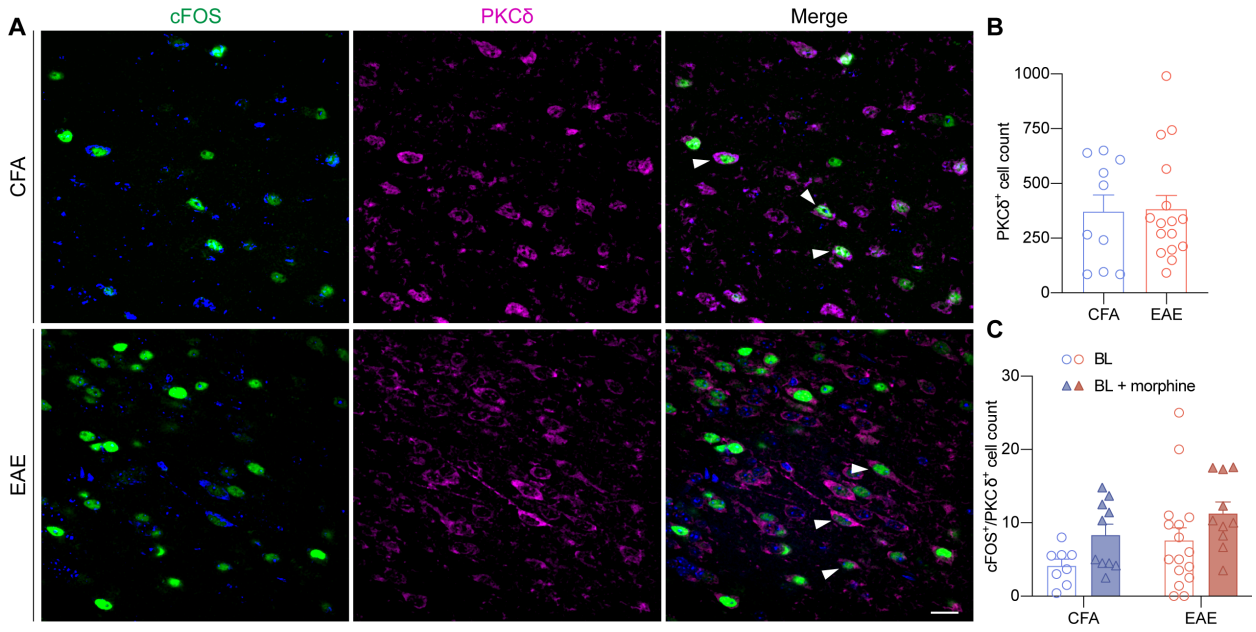


Figure 3-7. ***Pronociceptive signaling in the CeA is not significantly altered in EAE.*** **(A)** Representative 40X photomicrographs of cFOS **(green)** and PKC*δ* **(magenta)** immunostaining in the central amygdala of CFA and EAE animals at disease onset. White arrowheads indicate co-labelled cFOS+/PKC*δ*+ neurons. Scale bar = 20 µm, applies to all images. **(B)** The total number of PKC*δ*+ cells in the central amygdala was not significantly different between CFA-treated control and EAE mice. **(C)** The number of co-labelled cFOS+/PKC*δ*+ cells in the central amygdala was not significantly different between control and EAE animals at baseline or with an acute morphine (5 mg/kg, i.p.) challenge. All pairwise post-hoc analyses by Sidak's multiple comparisons test. BL, baseline; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis. Data presented as mean ± SEM.

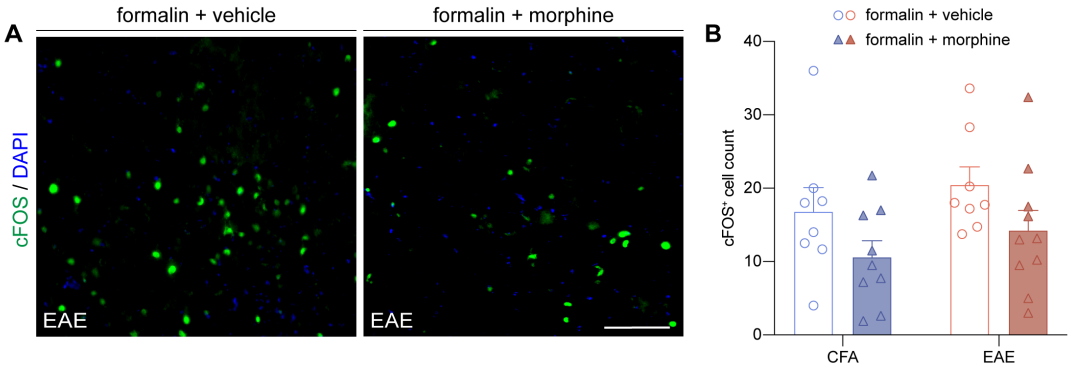


Figure 3-8. ***Formalin-evoked cFOS immunoreactivity in the CeA is not significantly altered in EAE.*** **(A)** Representative 20X photomicrographs of cFOS immunostaining in the central amygdala of EAE animals pretreated with saline vehicle or morphine (5 mg/kg, i.p.) on day of disease onset. Scale bar = 100 μm, applies to both images. **(B)** Formalin-evoked cFOS expression in the central amygdala was significantly reduced by morphine pretreatment in CFA and EAE mice. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

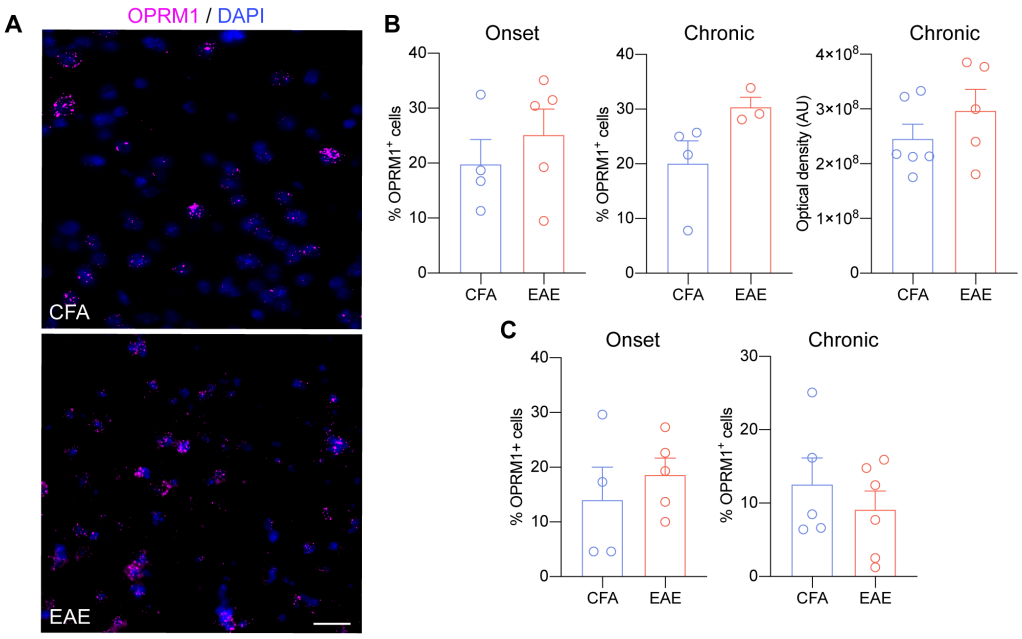


Figure 3-9. ***OPRM1 expression levels in the CeA and ITC cell mass are not significantly altered in EAE.*** **(A)** Representative 63X photomicrographs of *OPRM1* mRNA in the central amygdala detected with fluorescent *in situ* hybridization. Scale bar = 20 µm, applies to both images. **(B)** The percentage of *OPRM1*+ cells in the central amygdala was not significantly different between CFA control and EAE mice at disease onset (left) or in the chronic stage of disease (middle). Total central amygdala *OPRM1* expression did not differ between CFA and chronic EAE animals (right). **(C)** No significant differences were found in the percentage of *OPRM1*+ cells in the intercalated region between CFA controls and EAE animals at either stage of disease. Data presented as mean ± SEM. AU, arbitrary units; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

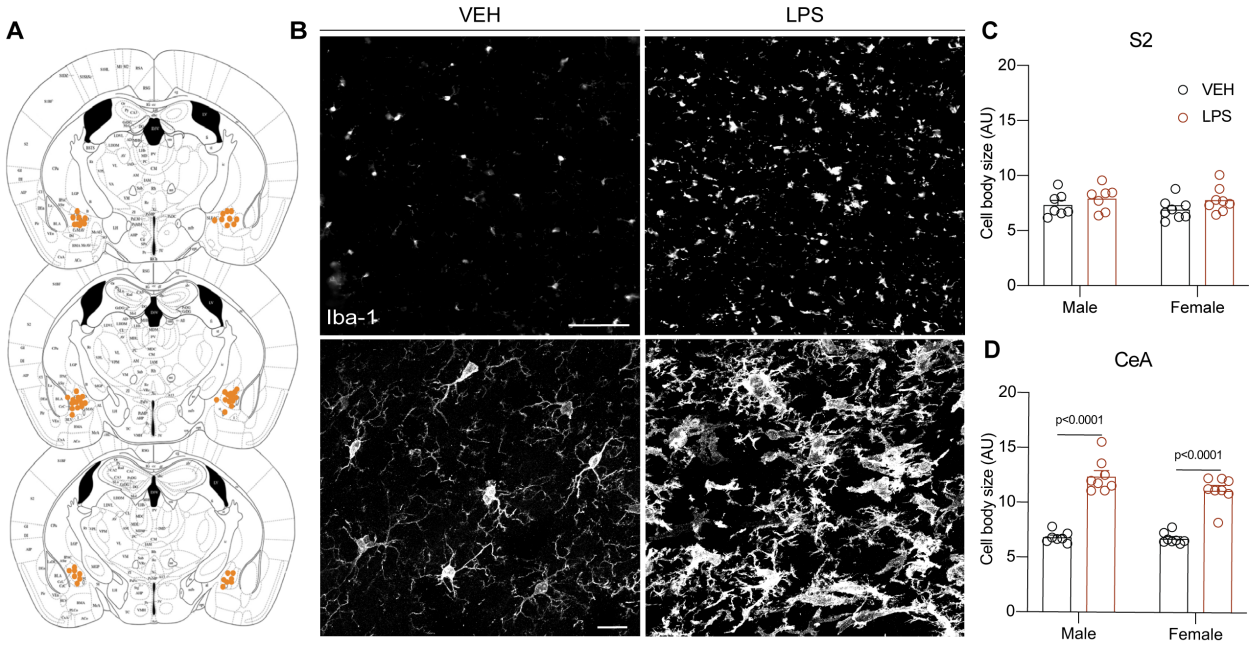


Figure 3-10. ***Intra-CeA injection of LPS induces focal microglial activation in males and females.*** **(A)** Representative schematic depicts stereotactic injection sites that targeted the central amygdala. **(B)** Representative immunomicrogaphs of Iba-1+ microglia in the central amygdala of vehicle- and LPS-treated animals at 20X (top panels, scale bar = 100 µm) and 63X magnification (bottom panels, scale bar = 20 µm). Quantification of Iba-1+ microglial cell body size in the **(C)** secondary sensory cortex and **(D)** central amygdala of LPS-treated mice compared to saline-treated controls. LPS-treated animals showed significant activation of microglia in the central amygdala. All pairwise post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM. AU, arbitrary units; CeA, central nucleus of the amygdala; LPS, lipopolysaccharide; S2, secondary sensory cortex; VEH, vehicle.

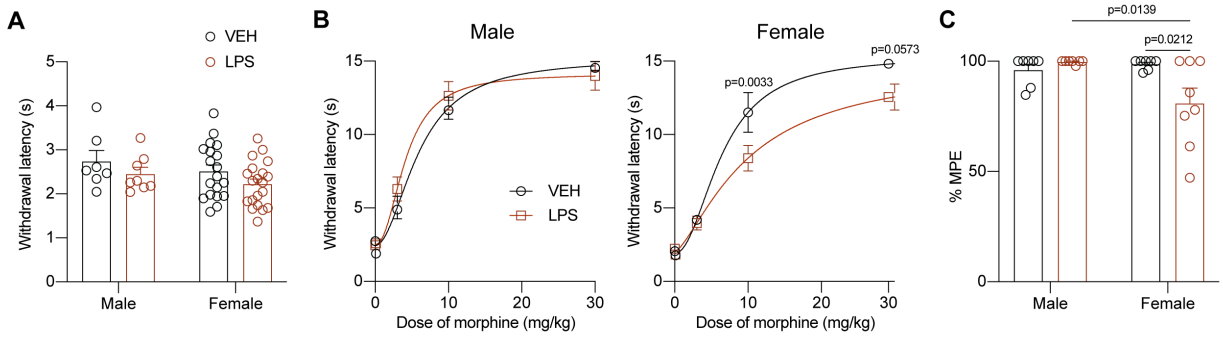
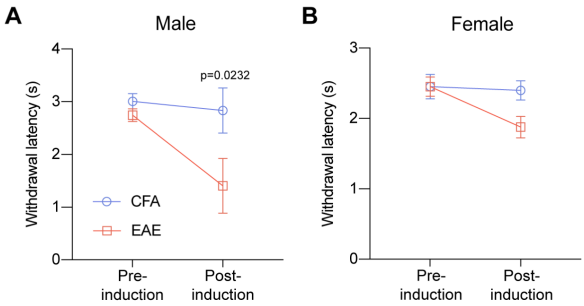
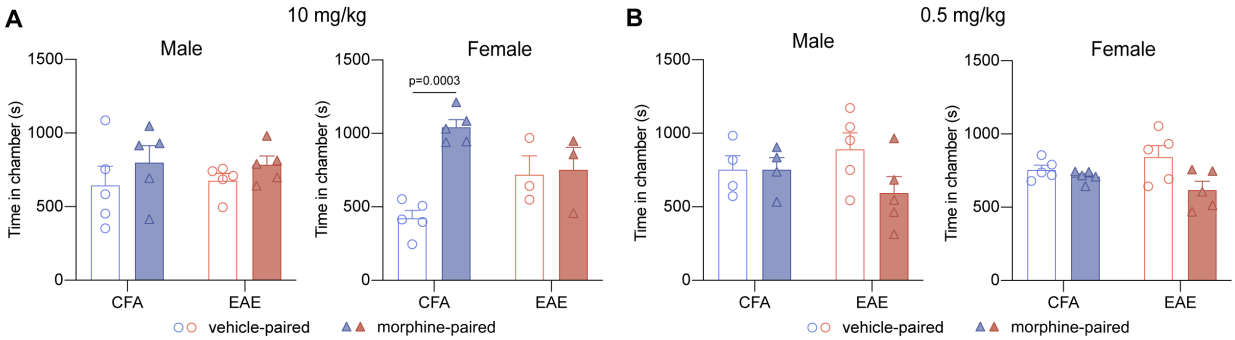


Figure 3-11. ***Microglial activation in the CeA attenuates morphine analgesia in females.*** **(A)** Baseline thermal tail withdrawal latencies were not significantly different between saline vehicle- and LPS-treated male and female mice. **(B)** Morphine (0.1-30 mg/kg) dose-response assayed using thermal tail withdrawal compared between male and female saline- and LPS-treated animals. Withdrawal latencies were not significantly different between saline- and LPS-treated males. Morphine antinociception was significantly reduced in female mice treated with LPS. **(C)** Transformed data (%MPE) of tail withdrawal thresholds from animals treated with morphine (30 mg/kg). All pairwise post-hoc analyses by Tukey's multiple comparisons test. Data presented as mean ± SEM. LPS, lipopolysaccharide; VEH, vehicle.

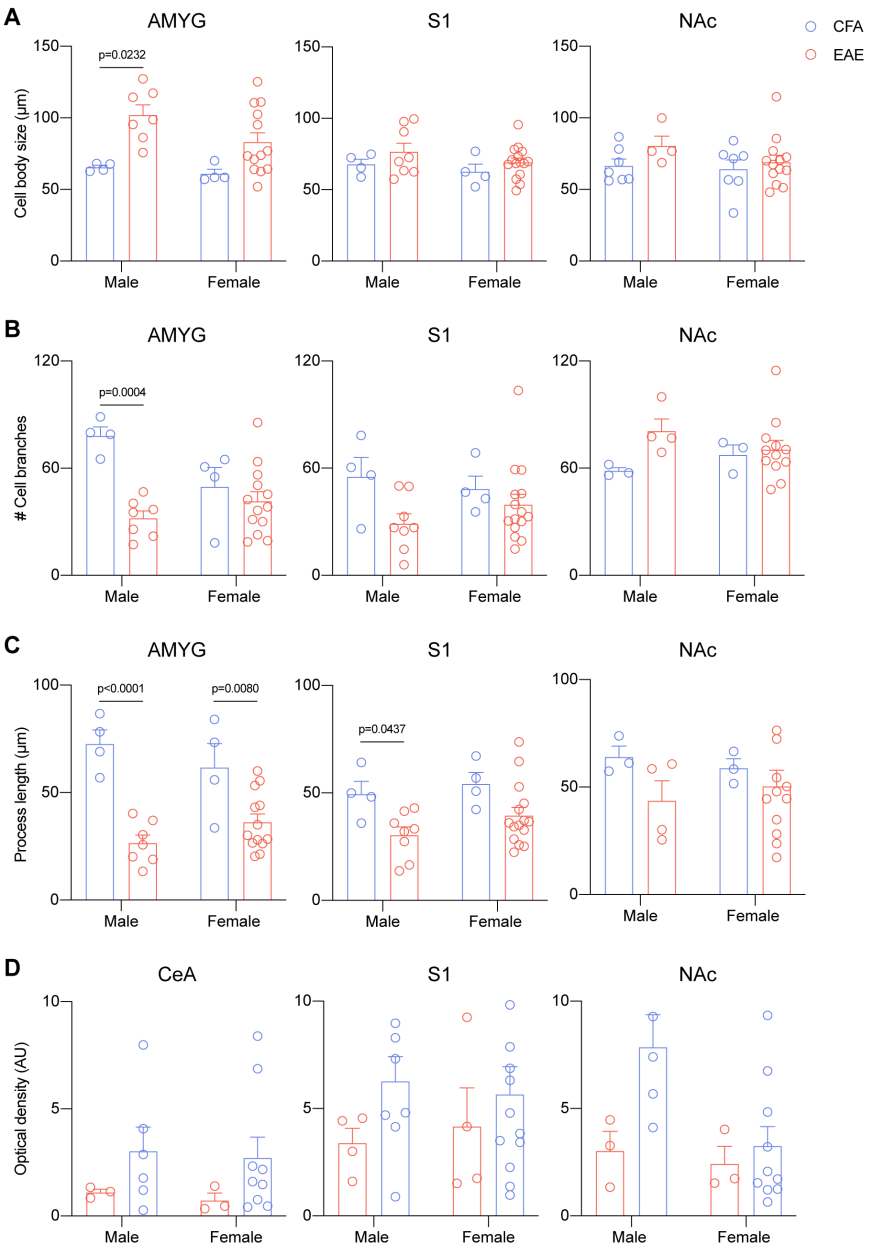
3.8 Supplementary Figures



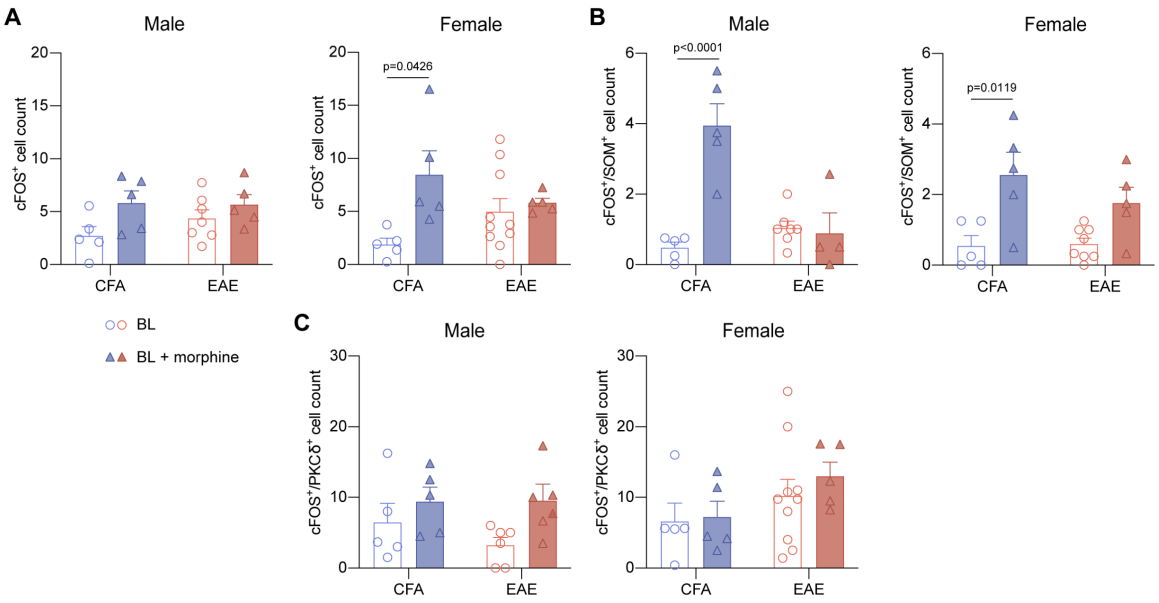
Supplementary Figure 3-1. ***No significant main effect of sex was found for baseline thermal tail withdrawal thresholds.*** **(A)** Male and **(B)** female mice with EAE displayed lower baseline thermal tail withdrawal thresholds than CFA-treated controls. Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.



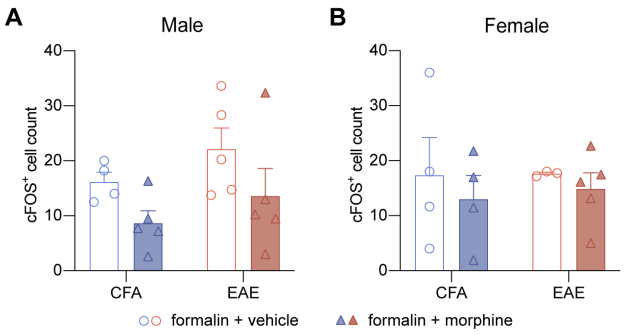
Supplementary Figure 3-2. ***No significant main effect of sex was found for morphine-induced conditioned place preference.*** **(A)** Systemic morphine (10 mg/kg) failed to produce a conditioned place preference in male and female mice with EAE. **(B)** Low-dose morphine (0.5 mg/kg) did not produce a place preference in CFA-treated controls and induced a place aversion in male and female EAE mice. Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.



Supplementary Figure 3-3. ***No significant main effect of sex was found for microglial morphology in the amygdala, S1, or NAc.*** **(A)** Cell body size, **(B)** number of cell branches, **(C)** mean process length, and **(D)** staining density of Iba-1+ microglia in the amygdala, primary somatosensory cortex (S1), and nucleus accumbens (NAc) were not significantly different between male and female CFA and EAE mice. Data presented as mean ± SEM. AMYG, amygdala; AU, arbitrary units; CeA, central amygdala; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis; NAc, nucleus accumbens; S1, primary somatosensory cortex.



Supplementary Figure 3-4. ***No significant main effect of sex was found for cFOS expression or colocalization in the CeA.*** **(A)** No significant differences were noted in cFOS+ cell counts in the central amygdala between male and female CFA and EAE mice. cFOS colocalization with **(B)** SOM+ and **(C)** PKCδ+ neurons in the central amygdala was not significantly different between male and female CFA and EAE mice. Data presented as mean ± SEM. BL, baseline; CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.



Supplementary Figure 3-5. ***No significant main effect of sex was found for formalin-evoked cFOS immunoreactivity in the CeA.*** Formalin-evoked cFOS cell counts in the central amygdala did not differ between **(A)** male and **(B)** female CFA control and EAE mice when treated with saline vehicle or morphine (5 mg/kg, i.p.). Data presented as mean ± SEM. CFA, Complete Freund's Adjuvant; EAE, experimental autoimmune encephalomyelitis.

CHAPTER 4: Discussion

4.1 Summary of Results

This study investigates pro- and anti-nociceptive signaling in male and female MOG_{35-55}-induced EAE mice. I assessed nociceptive behaviour and evaluated opioid-induced analgesia and reward in the early stages of disease. I employed immunohistological methods to explore alterations in central inflammation and neuronal activity within the central amygdala. Here, I provide evidence that MOG_{35-55} EAE induces pathological changes to the central amygdala that contribute to altered nociceptive signaling and pain hypersensitivity in a sexually dimorphic manner.

4.2 Using the EAE model to study MS-related pain

Inflammation is a canonical feature of MS and EAE pathology. We have previously shown that inflammatory markers within the CNS are elevated early in the EAE disease course (Olechowski et al., 2009; Potter et al., 2016). Indeed, we found that mice with EAE exhibit robust microgliosis and infiltration of T-lymphocytes in the spinal cord prior to the onset of paralytic symptoms. Mechanical and cold allodynia are well-established sensory disturbances in the MOG_{35-55} EAE model that develop in early disease stages and persist throughout the course of the disease (Olechowski et al., 2009; Rodrigues et al., 2009). Changes in heat sensitivity have only been described during the chronic phase of MOG_{35-55}- (Lu et al., 2012) and $PLP_{139–151}$-induced EAE (Aicher et al., 2004; Sanna et al., 2017), as well as the Theiler's murine encephalomyelitis virus (TMEV) model of MS (Lynch et al., 2008). Contrary to previous work, we demonstrate that EAE animals displayed heat hyperalgesia in the pre-symptomatic stage of disease (**FIG. 3-2**). This is consistent with clinical reports that many MS patients display sensory abnormalities such as temperature sensitivity, that often precede motor deficits (Kalia and

O'Connor, 2005). The differences in thermal sensitivities between our study and previous work may be attributed to the different assays used (tail withdrawal vs. Hargreaves plantar test) or may reflect inherent discrepancies in pain sensitivity between the mouse strains and models (SJL/J vs. C57BL/6). Together, the present findings suggest that pathophysiological events that precede locomotor dysfunction, such as CNS inflammation, play an integral role in the abnormal pain response in EAE. Our results are an important extension of the existing work that validates the use of the EAE model to study MS-related pain.

4.3 Potential mechanisms of pain hypersensitivity and reduced opioid antinociception

4.3.1 Increased microglial reactivity and dysregulated descending pain modulation

Opioid analgesics often provide inadequate relief for MS and neuropathic pain patients, except at high doses that might enhance the risk for adverse side effects. Animal models of chronic pain show reduced opioid analgesia compared with controls (Arnér and Meyerson, 1988; Zurek et al., 2001; Kalman et al., 2002; Luger et al., 2002; Lynch et al., 2008; Chen et al., 2013). In line with previous work, we show that morphine-induced analgesia was significantly impaired in EAE animals in a sex-dependent manner (**FIG. 3-3**). We found robust activation of microglial cells in the amygdala that correlated with thermal hypersensitivity and loss of morphine efficacy in females. Activation of microglia in the CeA by LPS resulted in a loss of opioid-mediated analgesia in a similar manner to EAE animals. This effect was only found in females, again reminiscent of the EAE condition (**FIG. 3-11**). Our results indicate that microglia play a critical role in modulating nociceptive processing in the CeA. In particular, these findings suggest that microglia specifically impair nociceptive signaling in females.

We did not observe any significant changes in baseline thermal hypersensitivity in response to intra-CeA LPS treatment (**FIG. 3-11**). It is important to note that pain hypersensitivity was assessed exclusively at 72 hours post-LPS treatment and may not reflect behaviour at earlier or later time points. It is also possible that animals developed mechanical and/or cold hypersensitivity; however, only one sensory modality was included within the scope of this study. Our data suggest that the observed loss of morphine antinociception in EAE may not be due to the offsetting of analgesia by an increase in pain sensitivity, but instead reflects the action of endogenous anti-analgesic mechanisms.

Neuroinflammation and reactive gliosis are known to be key mediators in the development and maintenance of chronic pain. Activated microglia are particularly critical to pain pathophysiology in EAE and MS (Jack et al., 2005; Olechowski et al., 2009). There is a marked activation of microglial cells in both the spinal cord and brain over the course of the disease, contributing to pain hypersensitivity across various modalities (Gehrmann et al., 1993; Gray et al., 2008; Olechowski et al., 2009; Mifflin et al., 2017; Catuneanu et al., 2019). Our findings within the CeA complement a vast literature indicating that microglia throughout the CNS contribute to pain hypersensitivity and acute morphine anti-analgesia. Glial inhibitors can improve pain symptoms in a variety of chronic pain conditions (Coull et al., 2005; Tozaki-Saitoh et al., 2008; Taylor et al., 2015). Moreover, microglial activation induced by neuropathic pain, persistent inflammatory pain, or intraperitoneally administered LPS attenuates the analgesic efficacy of acute morphine (Raghavendra et al., 2002; Johnston and Westbrook, 2005; Doyle et al., 2017). This effect is prevented by blocking microglial activation with systemic or intrathecal glia inhibitors (Raghavendra et al., 2002, 2003a; Johnston and Westbrook, 2003, 2005; Eidson and Murphy, 2013). It is clear that the physiological mechanisms that underlie pain and analgesia

are inextricably linked. While much of this previous work has been limited to spinal loci, we indicate a role for supraspinal microglial cell activity as well. The converging evidence of this study and previous research supports the notion that modulation of microglial and neuroimmune activation may be a therapeutic target for the enhancement of morphine analgesia and pain reduction. However, the development of novel therapies to manage MS-related pain in humans is unlikely to target specific brain regions and will likely involve systemic drug administration. The ability of systemically administered microglia-specific inhibitors to restore nociceptive signaling in the CeA and alleviate MS-related pain should be evaluated in future studies.

Recent research indicates that nerve-injury enhances excitability of PKCδ+ neurons in the CeA, contributing to pain-related behaviours (Wilson et al., 2019). Here, we found that CeA-PKCδ neurons displayed increased markers of cellular activation in EAE animals, though no significance was detected (**FIG. 3-7**). Conversely, CeA neurons expressing SOM contribute to an endogenous analgesic tone and display suppressed excitability during nerve injury-induced chronic pain. Chemogenetic activation of CeA-SOM cells results in robust analgesia that reverses this hypersensitivity (Wilson et al., 2019). The role of these neurons in modulating exogenous opioid-induced analgesia had not yet been established. In the present work, systemic morphine evoked a dramatic increase in the activity of CeA-SOM neurons and was associated with analgesia in CFA-treated control animals (**FIG. 3-6**). Compared with controls, SOM+ cells in EAE were less responsive to systemic morphine at disease onset, a time point which correlates with peak pain hypersensitivity (Olechowski et al., 2009). The consistent inhibition of CeA-SOM neurons in both central inflammatory (**FIG. 3-6**) and neuropathic (Wilson et al., 2019) pain states strongly points to their physiological relevance in the modulation of analgesic signals

emerging from the CeA. Our findings suggest that desensitization of SOM-expressing neurons in the CeA contributes to pain hypersensitivity and deficits in opioid analgesia in EAE.

The source of inhibition of the CeA-SOM cells in the context of EAE is unknown. My thesis work provides the first evidence that reactive gliosis contributes to this altered nociceptive processing in the CeA. It is well-established that glial cells are involved in the regulation of neuronal activity. In various brain pathologies and pain states, the pathological activation of microglia and dysfunction of neuronal activity are strongly correlated (Watkins et al., 2007; Béchade et al., 2013). For instance, activated microglia throughout the neural axis have been shown to release brain-derived neurotrophic factor (BDNF) and disrupt chloride transport in GABAergic interneurons, which are critical for maintaining normal nociceptive processing. This disinhibition contributes to hyperexcitability of the pain pathway and impaired responsivity to opioids (Coull et al., 2005; Trang et al., 2009; Gagnon et al., 2013; Taylor et al., 2015). Based on our findings, we hypothesize that activated microglia within the amygdala alter neurotransmission and reduce GABA-mediated inhibition of CeA-SOM cell projecting neurons, resulting in loss of descending antinociceptive activity and enhanced pain hypersensitivity. The results generated in this thesis can be used to guide future experiments targeting select cytokines that may mediate the disrupted balance between pro- and antinociceptive signaling differentially in males and females.

4.3.2 Dysfunction of the endogenous opioid system

A vast body of clinical evidence suggests that opioids lack potent analgesic efficacy in chronic pain (Arnér and Meyerson, 1988; Kalman et al., 2002; Rowbotham et al., 2003). The precise mechanisms for reduced sensitivity to systemic opioids in chronic pain are largely

unclear, though extensive evidence from human neuroimaging and preclinical animal studies points to alterations in central opioid signaling (Jones et al., 1994; Willoch et al., 1999; Zubieta et al., 2001; Harris et al., 2007; Maarrawi et al., 2007; Klega et al., 2010; DosSantos et al., 2012; Thompson et al., 2018). Dysfunction of the endogenous opioid system is evidenced in a variety of chronic pain conditions, including fibromyalgia, chronic back pain, and central neuropathic pain (Jones et al., 1994; Willoch et al., 1999; Harris et al., 2007; Schrepf et al., 2016). Thus, impairment of endogenous opioid signaling may be a mechanism that critically contributes to the chronicity of pain in MS. From a clinical perspective, dysregulation of the opioid system might also help to explain the poor patient response to this class of analgesics (Kalman et al., 2002). Literature surrounding the contribution of the endogenous opioid system to pain hypersensitivity in MS and EAE is limited. Spinal cord mRNA levels of all three opioid receptors (MOR, DOR, and KOR) are significantly decreased in mice with $MOG_{35–55}$-induced EAE (Du et al., 2016). Similarly, mice infected with TMEV show reductions in spinal cord MOR mRNA compared to uninfected control mice, which correlates with development of thermal and mechanical hyperalgesia (Lynch et al., 2008). We report no significant differences in *OPRM1* expression in the CeA or ITC cells between CFA control and EAE animals (**FIG. 3-9**). However, the approach utilized suffers from the limitation that only mRNA expression was measured. Our results do not preclude the possibilities of changes in MOR expression at the protein level, receptor internalization or desensitization, nor altered expression in other brain regions that form connections with the amygdala. Any of these maladaptive changes in MOR function could be reflected in the clinical manifestation of a prolonged pain experience. Future studies targeting the function and expression of MOR may uncover the role of μ-opioid signaling in regulating pain behaviours in MS.

Human and animal studies provide converging lines of evidence implicating opioid peptides in the pathogenesis of MS and EAE. MS patients and EAE animals show decreased levels of endogenous opioid peptides, including β-endorphin and enkephalin in the cerebrospinal fluid and peripheral blood mononuclear cell (PBMC) samples (Panerai et al., 1994; Gironi et al., 2000; Ludwig et al., 2017). Injections of [Met^5]-enkephalin (Jankovic and Maric, 1987) as well as blockade of opioid receptors with low dose naltrexone increase production of endogenous opioids and attenuate the onset and progression of disease (Zagon et al., 2009; Ludwig et al., 2017). The role of circulating endogenous opioids in MS has primarily been evaluated in the context of immunity and disease progression, and the contribution to pain development and maintenance has yet to be elucidated. One preliminary study involving 40 MS patients found no effect of treatment with low-dose naltrexone on pain measures, despite elevations in PBMC β-endorphin levels (Gironi et al., 2008). However, the interpretation of these results is limited by the uncontrolled design of the study, as well as the small sample size. Changes in endogenous opioidergic signaling are likely significant contributors to MS- and EAE-related pain. As such, restoring opioid system function may be a viable target for novel analgesic drugs and therapeutics to manage pain in this disease. Further studies should be performed to understand the contribution of endogenous opioid ligands and receptors to pain in MS.

4.4 Impaired opioid reward in EAE

Adaptations within affective and motivational circuits contribute to the cellular and behavioural maladaptations associated with chronic pain (Elman et al., 2013; Taylor et al., 2015). Indeed, studies using fMRI reveal that MS patients with chronic pain show structural and functional alterations in brain regions involved in reward processing, which are associated with

impaired reward responsiveness and depression (Pardini et al., 2013; Seixas et al., 2016; Heitmann et al., 2020). In the present study, we found that high-dose morphine (10 mg/kg) reliably produced a place preference in CFA control animals but failed to elicit a CPP in mice with EAE, indicating disrupted affective and reward processing in the EAE model (**FIG. 3-4**). This finding is consistent with previous reports showing that opioids, including morphine, are less rewarding in animals with chronic pain (Ozaki et al., 2002, 2003; Martin et al., 2007; Petraschka et al., 2007; Niikura et al., 2008). In contrast, other studies have indicated that both low and high doses of opioids remain reinforcing in peripheral nerve injury pain models, likely through their analgesic effects (Cahill et al., 2013; Taylor et al., 2015). There are several methodological differences that may account for the discrepant results between the current study and previous literature. Primarily, EAE is a model of T-cell-mediated inflammation and is thus inherently and phenotypically different from peripheral nerve injury models of neuropathic pain. An alternative explanation may involve the protocols used for conditioning to morphine. We exposed animals to opioids over two conditioning sessions, whereas other studies only reported a significant place preference after eight morphine conditioning sessions (Cahill et al., 2013; Taylor et al., 2015). Together, our results demonstrate that opioid-induced reward is disrupted in male and female mice with EAE, warranting further investigation into how EAE-induced pain alters motivation and reward circuitry.

There are complex interactions and extensive overlap between the neural circuitry of pain and reward processing (Leknes and Tracey, 2008). The amygdala, for example, is particularly well-situated to mediate interactions between pain and reward. We can therefore speculate that dysfunction within the amygdala would contribute to impairments in both pain processing and reward behaviour in EAE. Surprisingly, EAE animals exhibited a place aversion induced by low-

dose morphine (0.5 mg/kg) **(FIG. 3-4**). This result indicates that EAE mice retained the capacity for associative learning and suggests changes in dose-response sensitivity to morphine reward, although the implications for this observation remain elusive. In addition to analgesic properties, MOR agonists often lead to substantial dysphoric effects such as nausea, constipation, and respiratory depression (Furlan et al., 2006; Benyamin et al., 2008). Healthy, pain-free individuals treated with μ-opioid agonists including morphine (Zacny and Lichtor, 2008), oxycodone (Wardle et al., 2014) or remifentanil often report disliking the drug effects and dysphoria (Crozier et al., 2004; Wagner et al., 2010). Considering the low dose of morphine administered to EAE animals, the analgesic effects were likely minimal (if any) and lacked the analgesic potency to offset ongoing pain and the adverse effects of morphine. Thus, the dysphoric effects may have become more discernable, driving place aversion.

4.5 Potential mechanisms driving the sexually dimorphic effects of morphine

Here, we show that morphine-induced analgesia was significantly impaired in EAE animals in a sex-dependent manner. In EAE female, but not male mice, morphine analgesia was dramatically reduced compared to controls (**FIG. 3-3**). These results are in line with previous reports indicating sex differences in opioid analgesia (Fillingim and Gear, 2004; Craft, 2008; Bodnar and Kest, 2009; Bartley and Fillingim, 2013). Male rodents generally display significantly greater analgesic responses to systemically administered μ-opioid agonists than females. Enhanced sensitivity to morphine analgesia exhibited by male animals has been documented with several pain assays, including those assessing thermal (Candido et al., 1992; Cicero et al., 1996), chemical (Cicero et al., 1996), and visceral (Baamonde et al., 1989) nociception. In addition, female mice infected with TMEV display a significant decrease in

morphine-induced antinociception at a much earlier time point than males, despite similar reductions in spinal mRNA levels of opioid receptors (Lynch et al., 2008). Our results build on this previous literature and demonstrate that females exhibit an even greater reduction in opioid analgesic efficacy in chronic pain.

Human studies on the interaction of sex and opioid analgesia yield mixed research results. In contrast to rodents, there is some evidence that women respond more efficaciously than men to opioid analgesics (Sarton et al., 2000; Fillingim and Gear, 2004). The lack of consistent sex differences in opioid analgesia may reflect differences in methodology (e.g. species, model, quantification of analgesia). The presence or absence of sex differences in opioid antinociception in humans likely depends on several interacting variables, including sex hormones, psychosocial contexts, and the pharmacologic profile of the opioid used.

4.5.1 Dimorphisms in descending modulatory pathways

That we identified sex differences in morphine-induced analgesia, but not opioid reward, suggests divergent neural pathways of antinociception and reward, or sex-specific disease mechanisms. While both males and females demonstrated a similar impairment in opioid-induced CeA-SOM activation, only females exhibited a reduction in morphine analgesia. It is therefore likely that this sexual dimorphism in opioid analgesia reflects differences in signaling pathways downstream to the amygdala. The amygdala is involved in mediating descending pain modulation through projections to the PAG, which communicates with the RVM to project to the spinal cord to inhibit pain signals (Rizvi et al., 1991; Sun and Cassell, 1993; Pavlovic and Bodnar, 1998). The physiological organization and the functional activation of the CeA-PAG-RVM pathway are sexually distinct (Loyd and Murphy, 2006; Loyd et al., 2008; Cantu et al.,

2019). Female rats have significantly more PAG-RVM output neurons than males, whereas this circuit is preferentially engaged in males during persistent inflammatory pain states (Loyd and Murphy, 2006). Systemic morphine activates the descending modulatory pathway to a greater degree in males than females (Loyd and Murphy, 2006; Loyd et al., 2008). Recent work further indicates that activated microglia in the PAG contribute to sex differences in morphine antinociception. Specifically, increased activation of PAG microglia in female rats was found to contribute to an attenuated response to morphine (Doyle et al., 2017). Sex differences in the functional organization of this circuit may provide an anatomical basis and central mechanism for the sexually dimorphic effects of morphine in EAE.

Previous research has described differences in the microglial regulation of pain between the sexes, such that spinal microglia contribute to pain hypersensitivity particularly in males (Tomassini and Pozzilli, 2006; Sorge et al., 2011, 2015; Taves et al., 2015). However, this male-dependent microglial effect on pain is confined to the spinal cord. Intrathecal LPS injection elicits pain only in male mice, whereas administration to the brain and hindpaw produces equivalent mechanical allodynia in males and females. Importantly, LPS induces a similar level of spinal microgliosis in both sexes (Sorge et al., 2011). These findings indicate that sex differences in spinal microglial function and signaling, not in the degree of activation or proliferation, contribute to this sexually dimorphic role of microglia in pain. This aligns with our current study which describes similar levels of microglial activation in the CeA between male and female EAE mice (**FIG. 3-5**). A clear limitation of the present study is that only changes in microglial morphology and density were assessed. Future studies that examine microglial cell function and signaling are needed to dissect out the sexually distinct effects of microglial activation in EAE and determine how this contributes to CeA activity and pain behavior.

4.5.2 Sex hormones and chromosomes

The observed sex-dependent response to morphine in EAE may be attributed to the influence of gonadal hormones and/or sex chromosomes during development or adulthood. Studies involving the manipulation of gonadal hormones have provided strong evidence for the contribution of hormonal modulation to sexual dimorphisms in opioid analgesia (Stoffel et al., 2005; Bernal et al., 2007). Gonadal hormones are also implicated in differences in the progression of MS and EAE between the sexes (Polanczyk et al., 2004; Hoffman et al., 2006; Tomassini and Pozzilli, 2006). The complement of genes expressed on the X or Y chromosome contributes to sexually dimorphic phenotypes (Arnold, 2017). Indeed, sex chromosome complement is implicated in sex differences in nociception, as well as opioid analgesia in rodents (Gioiosa et al., 2008; Taylor et al., 2020). Future investigations are required to elucidate the influence of gonadal hormones and sex chromosomes on the sexually dimorphic analgesic effects of opioids in EAE.

4.6 Conclusion

This study offers novel insight on the role of the CeA in pain modulation in the EAE model. We show that CeA activity following nociceptive and analgesic stimuli is altered in mice with EAE in a sex-dependent manner. Though the precise mechanism of how pain and analgesia are differentially driven between the sexes remains to be determined, we identify microglial activation within the CeA as a key mediator in this dysfunction. Conventional pain management strategies provide inadequate relief for MS-related pain. Therefore, the development of novel therapies is necessary to effectively manage pain in this disease. Results from this study provide mechanistic insight into why opioids are ineffective at treating chronic pain in MS and suggest

that blocking microglial activation and restoring nociceptive signaling are viable targets for improving pain management in this patient population.

References

Acharjee S, Nayani N, Tsutsui M, Hill MN, Ousman SS, Pittman QJ (2013) Altered cognitive-emotional behavior in early experimental autoimmune encephalitis-Cytokine and hormonal correlates. Brain Behav Immun 33:164–172.

Acharjee S, Verbeek M, Gomez CD, Bisht K, Lee B, Benoit L, Sharkey KA, Benediktsson A, Tremblay ME, Pittman QJ (2018) Reduced microglial activity and enhanced glutamate transmission in the basolateral amygdala in early CNS autoimmunity. J Neurosci 38:9019–9033.

Adedoyin MO, Vicini S, Neale JH (2010) Endogenous N-acetylaspartylglutamate (NAAG) inhibits synaptic plasticity/transmission in the amygdala in a mouse inflammatory pain model. Mol Pain 6:1744-8069-6–60.

Aicher SA, Silverman MB, Winkler CW, Bebo BF (2004) Hyperalgesia in an animal model of multiple sclerosis. Pain 110:560–570.

Al-Hasani R, Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology 115:1363–1381.

Annese J, Schenker-Ahmed NM, Bartsch H, Maechler P, Sheh C, Thomas N, Kayano J, Ghatan A, Bresler N, Frosch MP, Klaming R, Corkin S (2014) Postmortem examination of patient H.M.'s brain based on histological sectioning and digital 3D reconstruction. Nat Commun 5:1–9.

Apkarian AV, Hashmi JA, Baliki MN (2011) Pain and the brain: Specificity and plasticity of the brain in clinical chronic pain. Pain 152:S64.

Arai H, Furuya T, Yasuda T, Miura M, Mizuno Y, Mochizuki H (2004) Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1, and expression of caspase-11 in mice.

Arnér S, Meyerson BA (1988) Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. Pain 33:11–23.

Arnold AP (2017) A general theory of sexual differentiation. J Neurosci Res 95:291–300.

Baamonde AI, Hidalgo A, Andrés-Trelles F (1989) Sex-related differences in the effects of morphine and stress on visceral pain. Neuropharmacology 28:967–970.

Bachiller S, Jiménez-Ferrer I, Paulus A, Yang Y, Swanberg M, Deierborg T, Boza-Serrano A (2018) Microglia in neurological diseases: A road map to brain-disease dependent-inflammatory response. Front Cell Neurosci 12:488.

Baliki MN, Chialvo DR, Geha PY, Levy RM, Harden RN, Parrish TB, Apkarian AV (2006) Chronic pain and the emotional brain: Specific brain activity associated with spontaneous fluctuations of intensity of chronic back pain.

Baliki MN, Geha PY, Jabakhanji R, Harden N, Schnitzer TJ, Apkarian AV (2008) A preliminary fMRI study of analgesic treatment in chronic back pain and knee osteoarthritis. Mol Pain 4:47.

Baliki MN, Petre B, Torbey S, Herrmann KM, Huang L, Schnitzer TJ, Fields HL, Apkarian AV (2012) Corticostriatal functional connectivity predicts transition to chronic back pain. Nat Neurosci 15:1117–1119.

Barcelon EE, Cho W-H, Jun SB, Lee SJ (2019) Brain microglial activation in chronic pain-associated affective disorder. Front Neurosci 13:1–10.

Bartley EJ, Fillingim RB (2013) Sex differences in pain: A brief review of clinical and experimental findings. Br J Anaesth 111:52–58.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267–284.

Basbaum AI, Fields HL (1984) Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci VOL. 7:309–338.

Béchade C, Cantaut-Belarif Y, Bessis A (2013) Microglial control of neuronal activity. Front Cell Neurosci 7:32.

Beggs S, Salter MW (2007) Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. Brain Behav Immun 21:624–633.

Beggs S, Trang T, Salter MW (2012) P2X4R+ microglia drive neuropathic pain. Nat Neurosci 15:1068–1073.

Benarroch EE (2012) Endogenous opioid systems: Current concepts and clinical correlations. Neurology 79:807–814.

Bencherif B, Fuchs PN, Sheth R, Dannals RF, Campbell JN, Frost JJ (2002) Pain activation of human supraspinal opioid pathways as demonstrated by [11C]-carfentanil and positron emission tomography (PET). Pain 99:589–598.

Bennett DLH, Woods CG (2014) Painful and painless channelopathies. Lancet Neurol 13:587–599.

Benyamin R, Trescot A., Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R (2008) Opioid complications and side effects. Pain Physician:S105–S120.

Berkley KJ (1997) Sex differences in pain. Behav Brain Sci 20:371–380.

Bernal SA, Morgan MM, Craft RM (2007) PAG mu opioid receptor activation underlies sex differences in morphine antinociception. Behav Brain Res 177:126–133.

Bernard JF, Besson JM (1990) The spino(trigemino)pontoamygdaloid pathway:

Electrophysiological evidence for an involvement in pain processes. J Neurophysiol 63:473–490.

Bernard JF, Huang GF, Besson JM (1992) Nucleus centralis of the amygdala and the globus pallidus ventralis: Electrophysiological evidence for an involvement in pain processes. J Neurophysiol 68:551–569.

Bird GC, Lash LL, Han JS, Zou X, Willis WD, Neugebauer V (2005) Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. J Physiol 564:907–921.

Blaesse P, Goedecke L, Bazelot M, Capogna M, Pape HC, Jüngling K (2015) μ-Opioid receptor-mediated inhibition of intercalated neurons and effect on synaptic transmission to the central amygdala. J Neurosci 35:7317–7325.

Bodnar RJ, Kest B (2009) Sex differences in opioid analgesia, hyperalgesia, tolerance and withdrawal: Central mechanisms of action and roles of gonadal hormones. Horm Behav 58:72–81.

Bourgeais L, Gauriau C, Bernard JF (2001) Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: A PHA-L study in the rat. Eur J Neurosci 14:229–255.

Braz JM, Nassar MA, Wood JN, Basbaum AI (2005) Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron 47:787–793.

Burstein R, Potrebic S (1993) Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. J Comp Neurol 335:469–485.

Bushnell MC, Čeko M, Low LA (2013) Cognitive and emotional control of pain and its disruption in chronic pain. Nat Rev Neurosci 14:502–511.

Cahill CM, Xue L, Grenier P, Magnussen C, Lecour S, Olmstead MC (2013) Changes in morphine reward in a model of neuropathic pain. Behav Pharmacol 24:207–213.

Candido J, Lutfy K, Billings B, Sierra V, Duttaroy A, Inturrisi CE, Yoburn BC (1992) Effect of adrenal and sex hormones on opioid analgesia and opioid receptor regulation. Pharmacol Biochem Behav 42:685–692.

Cantu DJ, Kaur S, Murphy AZ, Averitt DL (2019) Sex differences in the amygdaloid projections to the periaqueductal gray during inflammatory pain in the rat. FASEB J 33:448.3-448.3.

Carrasquillo Y, Gereau IV RW (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. J Neurosci 27:1543–1551.

Carrasquillo Y, Gereau IV RW (2008) Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. Mol Pain 4:1744-8069-4–24.

Castaño A, Herrera AJ, Cano J, Machado A (1998) Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem 70:1584–1592.

Catuneanu A, Paylor JW, Winship I, Colbourne F, Kerr BJ (2019) Sex differences in central nervous system plasticity and pain in experimental autoimmune encephalomyelitis. Pain 160:1037–1049.

Chang P-C, Centeno MV, Procissi D, Baria A, Apkarian AV (2017) Brain activity for tactile allodynia. Pain 158:488–497.

Chen G, Luo X, Qadri MY, Berta T, Ji RR (2018) Sex-dependent glial signaling in pathological pain: Distinct roles of spinal microglia and astrocytes. Neurosci Bull 34:98–108.

Chen L, Vo T, Seefeld L, Malarick C, Houghton M, Ahmed S, Zhang Y, Cohen A, Retamozo C, St. Hilaire K, Zhang V, Mao J (2013) Lack of correlation between opioid dose adjustment and pain score change in a group of chronic pain patients. J Pain 14:384–392.

Chen MJ, Kress B, Han X, Moll K, Peng W, Ji R-R, Nedergaard M (2012) Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. Glia 60:1660–1670.

Cheng S-J, Chen C-C, Yang H-W, Chang Y-T, Bai S-W, Chen C-C, Yen C-T, Min M-Y (2011) Role of extracellular signal-regulated kinase in synapticc transmission and plasticity of a nociceptive input on capsular central amygdaloid neurons in normal and acid-induced muscle pain. J Neurosci 31:2258–2270.

Cicero TJ, Nock B, Meyer EA (1996) Gender-related differences in the antinociceptive properties of morphine. J Pharmacol Exp Ther 279:767–773.

Coderre TJ, Fundytus ME, Mckenna JE, Dalal S, Melzack R (1993) The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain 54:43–50.

Compston A, Coles A (2008) Multiple sclerosis. Lancet 372:1502–1517.

Corbett AD, Henderson G, McKnight AT, Paterson SJ (2006) 75 Years of opioid research: The exciting but vain quest for the Holy Grail. Br J Pharmacol 147:S153.

Corder G, Ahanonu B, Grewe BF, Wang D, Schnitzer MJ, Scherrer G (2019) An amygdalar neural ensemble that encodes the unpleasantness of pain. Science (80-) 363:276–281.

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

Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes

congenital inability to experience pain. Nature 444:894–898.

Coyle DE (1998) Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. Glia 23:75–83.

Craft R (2008) Sex differences in analgesic, reinforcing, discriminative, and motoric effects of opioids. Exp Clin Psychopharmacol 16:376–385.

Crock LW, Kolber BJ, Morgan CD, Sadler KE, Vogt SK, Bruchas MR, Gereau Iv RW (2012) Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain.

Crown ED, King TE, Meagher MW, Grau JW (2000) Shock-induced hyperalgesia: III. Role of the bed nucleus of the stria terminalis and amygdaloid nuclei. Behav Neurosci 114:561–573.

Crozier T., Kietzmann D, Döbereiner B (2004) Mood change after anaesthesia with remifentanil or alfentanil. J Anaesthesiol 21:20–24.

D'amour FE, Smith DL (1941) A method for determining loss of pain sensation. J Pharmacol Exp Ther.

Da Costa Gomez TM, Behbehani MM (1995) An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. Brain Res 689:21–31.

Davis M (1998) Are different parts of the extended amygdala involved in fear versus anxiety? Biol Psychiatry 44:1239–1247.

Diaz-Olavarrieta C, Cummings JL, Velazquez J, Garcia De Al Cadena C (1999) Neuropsychiatric manifestations of multiple sclerosis. J Neuropsychiatry Clin Neurosci 11:51–57.

Dossani RH, Missios S, Nanda A (2015) The legacy of Henry Molaison (1926-2008) and the impact of his bilateral mesial temporal lobe surgery on the study of human memory. World Neurosurg 84:1127–1135.

DosSantos MF, Martikainen IK, Nascimento TD, Love TM, Deboer MD, Maslowski EC, Monteiro AA, Vincent MB, Zubieta JK, DaSilva AF (2012) Reduced basal ganglia μ-opioid receptor availability in trigeminal neuropathic pain: A pilot study. Mol Pain 8:1744-8069-8–74.

Doyle HH, Eidson LN, Sinkiewicz DM, Murphy AZ (2017) Sex differences in microglia activity within the periaqueductal gray of the rat: A potential mechanism driving the dimorphic effects of morphine. J Neurosci 37:3202–3214.

Du C, Duan Y, Wei W, Cai Y, Chai H, Lv J, Du X, Zhu J, Xie X (2016) Kappa opioid receptor activation alleviates experimental autoimmune encephalomyelitis and promotes oligodendrocyte-mediated remyelination. Nat Commun 7:1–10.

Dubin AE, Patapoutian A (2010) Nociceptors: The sensors of the pain pathway. J Clin Invest 120:3760–3772.

Dueñas M, Ojeda B, Salazar A, Mico JA, Failde I (2016) A review of chronic pain impact on patients, their social environment and the health care system. J Pain Res 9:457–467.

Ebersberger A, Anton F, Tölle TR, Zieglgänsberger W (1995) Morphine, 5-HT2 and 5-HT3 receptor antagonists reduce c-fos expression in the trigeminal nuclear complex following noxious chemical stimulation of the rat nasal mucosa. Brain Res 676:336–342.

Eidson LN, Murphy AZ (2013) Blockade of toll-like receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of morphine. J Neurosci 33:15952–15963.

Elman I, Borsook D, Volkow ND (2013) Pain and suicidality: Insights from reward and addiction neuroscience. Prog Neurobiol 109:1–27.

Ferrini F, De Koninck Y (2013) Microglia control neuronal network excitability via BDNF signalling. Neural Plast 2013:11.

Fillingim RB, Gear RW (2004) Sex differences in opioid analgesia: Clinical and experimental findings. Eur J Pain 8:413–425.

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

Finnegan TF, Chen SR, Pan HL (2005) Effect of the μ opioid on excitatory and inhibitory synaptic inputs to periaqueductal gray-projecting neurons in the amygdala. J Pharmacol Exp Ther 312:441–448.

Finnerup NB et al. (2015) Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. Lancet Neurol 14:162–173.

Fox RJ, Sorenson CA (1994) Bilateral lesions of the amygdala attenuate analgesia induced by diverse environmental challenges. Brain Res 648:215–221.

Fu Y, Neugebauer V (2008) Differential mechanisms of CRF1 and CRF2 receptor functions in the amygdala in pain-related synaptic facilitation and behavior. J Neurosci 28:3861–3876.

Furlan AD, Sandoval JA, Mailis-Gagnon A, Tunks E (2006) Opioids for chronic noncancer pain: A meta-analysis of effectiveness and side effects. CMAJ 174:1589–1594.

Gagnon M, Bergeron MJ, Lavertu G, Castonguay A, Tripathy S, Bonin RP, Perez-Sanchez J, Boudreau D, Wang B, Dumas L, Valade I, Bachand K, Jacob-Wagner M, Tardif C, Kianicka I, Isenring P, Attardo G, Coull JAM, De Koninck Y (2013) Chloride extrusion enhancers as novel therapeutics for neurological diseases. Nat Med 19:1524–1528.

Gao Y-J, Ji R-R (2009) c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? Open Pain J 2:11–17.

Gauriau C, Bernard JF (2002) Pain pathways and parabrachial circuits in the rat. Exp Physiol 87:251–258.

Gehrmann J, Gold R, Linington C, Lannes-Vieira J, Wekerle H, Kreutzberg GW (1993) Microglial involvement in experimental autoimmune inflammation of the central and peripheral nervous system. Glia 7:50–59.

Gilmour H, Ramage-Morin PL, Wong SL (2018) Multiple sclerosis: Prevalence and impact. Stat Canada Heal Reports 29:3–8.

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

Gironi M, Martinelli-Boneschi F, Sacerdote P, Solaro C, Zaffaroni M, Cavarretta R, Moiola L, Bucello S, Radaelli M, Pilato V, Cursi M, Franchi S, Martinelli V, Nemni R, Comi G, Martino G (2008) A pilot trial of low-dose naltrexone in primary progressive multiple sclerosis. Mult Scler 14:1076–1083.

Gironi M, Martinelli V, Brambilla E, Furlan R, Panerai AE, Comi G, Sacerdote P (2000) β-Endorphin concentrations in peripheral blood mononuclear cells of patients with multiple sclerosis: Effects of treatment with interferon beta. Arch Neurol 57:1178–1181.

Gold MS, Gebhart GF (2010) Nociceptor sensitization in pain pathogenesis. Nat Med 16:1248–1257.

Goldberg DS, McGee SJ (2011) Pain as a global public health priority. BMC Public Health 11:770.

Golden LC, Voskuhl R (2017) The importance of studying sex differences in disease: The example of multiple sclerosis. J Neurosci Res 95:633–643.

Gonçalves L, Dickenson AH (2012) Asymmetric time-dependent activation of right central amygdala neurones in rats with peripheral neuropathy and pregabalin modulation. Eur J Neurosci 36:3204–3213.

Gonçalves L, Silva R, Pinto-Ribeiro F, Pêgo JM, Bessa JM, Pertovaara A, Sousa N, Almeida A (2008) Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp Neurol 213:48–56.

Gray E, Thomas TL, Betmouni S, Scolding N, Love S (2008) Elevated activity and microglial expression of myeloperoxidase in demyelinated cerebral cortex in multiple sclerosis. Brain Pathol 18:86–95.

Gregoriou GC, Kissiwaa SA, Patel SD, Bagley EE (2019) Dopamine and opioids inhibit synaptic outputs of the main island of the intercalated neurons of the amygdala. Eur J Neurosci 50:2065–2074.

Grichnik KP, Ferrante FM (1991) The difference between acute and chronic pain. Mt Sinai J

Med 58:217–220.

Gu N, Peng J, Murugan M, Wang X, Eyo UB, Sun D, Ren Y, DiCicco-Bloom E, Young W, Dong H, Wu L-J (2016) Spinal microgliosis due to resident microglial proliferation is required for pain hypersensitivity after peripheral nerve injury. Cell Rep 16:605–614.

Gui WS, Wei X, Mai CL, Murugan M, Wu LJ, Xin WJ, Zhou LJ, Liu XG (2016) Interleukin-1β overproduction is a common cause for neuropathic pain, memory deficit, and depression following peripheral nerve injury in rodents. Mol Pain 12:1–15.

Hains BC, Waxman SG (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. J Neurosci 26:4308–4317.

Han JS, Adwanikar H, Li Z, Ji G, Neugebauer V (2010) Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. Mol Pain 6:1744–8069.

Han JS, Neugebauer V (2004) Synaptic plasticity in the amygdala in a visceral pain model in rats. Neurosci Lett 361:254–257.

Han JS, Neugebauer V (2005) mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain 113:211–222.

Hanisch U-K (2002) Microglia as a source and target of cytokines. Glia 40:140–155.

Hanken K, Francis Y, Kastrup A, Eling P, Klein J, Hildebrandt H (2018) On the role of the amygdala for experiencing fatigue in patients with multiple sclerosis. Mult Scler Relat Disord 20:67–72.

Harris RE, Clauw DJ, Scott DJ, McLean SA, Gracely RH, Zubieta JK (2007) Decreased central μ-opioid receptor availability in fibromyalgia. J Neurosci 27:10000–10006.

Hashmi JA, Baliki MN, Huang L, Baria AT, Torbey S, Hermann KM, Schnitzer TJ, Apkarian AV, Apkarian : A Vania (2013) Shape shifting pain: chronification of back pain shifts brain representation from nociceptive to emotional circuits. Brain 136:2751–2768.

Hebben N, Corkin S, Eichenbaum H, Shedlack K (1985) Diminished ability to interpret and report internal states after bilateral medial temporal resection: Case H.M. Behav Neurosci 99:1031–1039.

Heitmann H, Andlauer TFM, Korn T, Mühlau M, Henningsen P, Hemmer B, Ploner M (2020) Fatigue, depression, and pain in multiple sclerosis: How neuroinflammation translates into dysfunctional reward processing and anhedonic symptoms. Mult Scler J:135245852097227.

Helmstetter FJ (1992) The amygdala is essential for the expression of conditional hypoalgesia. Behav Neurosci 106:518–528.

Helmstetter FJ, Bellgowan PS (1993) Lesions of the amygdala block conditional hypoalgesia on

the tail flick test. Brain Res 612:253–257.

Hoffman GE, Merchenthaler I, Zup SL (2006) Neuroprotection by ovarian hormones in animal models of neurological disease. Endocrine 29:217–231.

Hogan M-E, Taddio A, Katz J, Shah V, Krahn M (2016) Incremental health care costs for chronic pain in Ontario, Canada. Pain 157:1626–1633.

Hua X-Y, Svensson CI, Matsui T, Fitzsimmons B, Yaksh TL, Webb M (2005) Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia. Eur J Neurosci 22:2431–2440.

Huang G-F, Besson J-M, Bernard J-F (1993) Intravenous morphine depresses the transmission of noxious messages to the nucleus centralis of the amygdala. Eur J Pharmacol 236:449.

Iannitti T, Kerr BJ, Taylor BK (2014) Mechanisms and pharmacology of neuropathic pain in multiple sclerosis. Curr Top Behav Neurosci 20:75–97.

Ikeda R, Takahashi Y, Inoue K, Kato F (2007) NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 127:161–172.

Inoue K, Tsuda M (2009) Microglia and neuropathic pain. Glia 57:1469–1479.

Jack C, Ruffini F, Bar-Or A, Antel JP (2005) Microglia and multiple sclerosis. J Neurosci Res 81:363–373.

Jankovic BD, Maric D (1987) Enkephalins and autoimmunity: Differential effect of methionine-enkephalin on experimental allergic encephalomyelitis in wistar and lewis rats. J Neurosci Res 18:88–94.

Jasmin L, Burkey AR, Patrick Card J, Basbaum AI (1997) Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. J Neurosci 17:3751–3765.

Jasmin L, Wang H, Tarczy-Hornoch K, Levine JD, Basbaum1~5~6 AI (1994) Differential effects of morphine on noxious stimulus-evoked Fos-like immunoreactivity in subpopulations of spinoparabrachial neurons.

Ji G, Neugebauer V (2011) Pain-related deactivation of medial prefrontal cortical neurons involves mGluR1 and GABA A receptors. J Neurophysiol 106:2642–2652.

Jiang H, Fang D, Kong LY, Jin ZR, Cai J, Kang XJ, Wan Y, Xing GG (2014) Sensitization of neurons in the central nucleus of the amygdala via the decreased GABAergic inhibition contributes to the development of neuropathic pain-related anxiety-like behaviors in rats. Mol Brain 7:72.

Johnston IN, Westbrook RF (2003) Acute and conditioned sickness reduces morphine analgesia. Behav Brain Res 142:89–97.

Johnston IN, Westbrook RF (2005) Inhibition of morphine analgesia by LPS: Role of opioid and NMDA receptors and spinal glia. Behav Brain Res 156:75–83.

Jones AKP, Cunningham VJ, Ha-Kawa^ S, Fujiwara T, Luthra SK, Silva S, Derbyshire S, Jones T (1994) Changes in central opioid receptor binding in relation to inflammation and pain in patients with rheumatoid arthritis. Br J Rheumatol 33:33–42.

Jones AKP, Watabe H, Cunningham VJ, Jones T (2004) Cerebral decreases in opioid receptor binding in patients with central neuropathic pain measured by [11C]diprenorphine binding and PET. Eur J Pain 8:479–485.

Jüngling K, Seidenbecher T, Sosulina L, Lesting J, Sangha S, Clark SD, Okamura N, Duangdao DM, Xu YL, Reinscheid RK, Pape HC (2008) Neuropeptide S-mediated control of fear expression and extinction: Role of intercalated GABAergic neurons in the amygdala. Neuron 59:298–310.

Kacimi R, Giffard RG, Yenari MA (2011) Endotoxin-activated microglia injure brain derived endothelial cells via NF-κB, JAK-STAT and JNK stress kinase pathways. J Inflamm 8:7.

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

Kalman S, Österberg AO, Sörensen J, Boivie J, Bertler A (2002) Morphine responsiveness in a group of well-defined multiple sclerosis patients: A study with i.v. morphine. Eur J Pain 6:69–80.

Keogh E, Bond FW, Hanmer R, Tilston J (2005) Comparing acceptance- and control-based coping instructions on the cold-pressor pain experiences of healthy men and women. Eur J Pain 9:591.

Keogh E, Herdenfeldt M (2002) Gender, coping and the perception of pain. Pain 97:195–201.

Khan N, Smith MT (2014) Multiple sclerosis-induced neuropathic pain: Pharmacological management and pathophysiological insights from rodent EAE models. Inflammopharmacology 22:1–22.

Kim J, Zhang X, Muralidhar S, LeBlanc SA, Tonegawa S (2017) Basolateral to central amygdala neural circuits for appetitive behaviors. Neuron 93:1464–1479.

Kipp M, van der Star B, Vogel DY, Puentes F, van der Valk P, Baker D, Amor S (2012) Experimental in vivo and in vitro models of multiple sclerosis: EAE and beyond. Mult Scler Relat Disord 1:15–28.

Klega A, Eberle T, Buchholz HG, Maus S, Maihöfner C, Schreckenberger M, Birklein F (2010) Central opioidergic neurotransmission in complex regional pain syndrome. Neurology 75:129–136.

Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau IV RW

(2010) Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior. J Neurosci 30:8203–8213.

Korostil M, Feinstein A (2007) Anxiety disorders and their clinical correlates in multiple sclerosis patients . Mult Scler J 13:67–72.

Kulkarni B, Bentley DE, Elliott R, Julyan PJ, Boger E, Watson A, Boyle Y, El-Deredy W, Jones AKP (2007) Arthritic pain is processed in brain areas concerned with emotions and fear. Arthritis Rheum 56:1345–1354.

LeDoux JE (2000) Emotion Circuits in the Brain. Annu Rev Neurosci 23:155–184.

Lee K, Vuong HE, Nusbaum DJ, Hsiao EY, Evans CJ, Taylor AMW (2018) The gut microbiota mediates reward and sensory responses associated with regimen-selective morphine dependence. Neuropsychopharmacology 43:2606–2614.

Leknes S, Tracey I (2008) A common neurobiology for pain and pleasure. Nat Rev Neurosci 9:314–320.

Li H, Penzo MA, Taniguchi H, Kopec CD, Huang ZJ, Li B (2013) Experience-dependent modification of a central amygdala fear circuit. Nat Neurosci 16:332–339.

Likhtik E, Popa D, Apergis-Schoute J, Fidacaro GA, Paré D (2008) Amygdala intercalated neurons are required for expression of fear extinction. Nature 454:642–645.

Liu CC, Ohara S, Franaszczuk P, Zagzoog N, Gallagher M, Lenz FA (2010) Painful stimuli evoke potentials recorded from the medial temporal lobe in humans. Neuroscience 165:1402–1411.

Loggia ML, Chonde DB, Akeju O, Arabasz G, Catana C, Edwards RR, Hill E, Hsu S, Izquierdo-Garcia D, Ji R-R, Riley M, Wasan AD, Zürcher NR, Albrecht DS, Vangel MG, Rosen BR, Napadow V, Hooker JM (2015) Evidence for brain glial activation in chronic pain patients. Brain 138:604–615.

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

Loyd DR, Wang X, Murphy AZ (2008) Sex differences in mu-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. J Neurosci 28:14007–14017.

Lu J, Kurejova M, Wirotanseng LN, Linker RA, Kuner R, Tappe-Theodor A (2012) Pain in experimental autoimmune encephalitis: A comparative study between different mouse models. J Neuroinflammation 9:233.

Lu YC, Chen YZ, Wei YY, He XT, Li X, Hu W, Yanagawa Y, Wang W, Wu SX, Dong YL (2014) Neurochemical properties of the synapses between the parabrachial nucleus-derived

CGRP-positive axonal terminals and the GABAergic neurons in the lateral capsular division of central nucleus of amygdala. Mol Neurobiol 51:105–118.

Lublin FD, Reingold SC (1996) Defining the clinical course of multiple sclerosis: Results of an international survey. Neurology 46:907–911.

Ludwig MD, Zagon IS, McLaughlin PJ (2017) Serum [Met5]-enkephalin levels are reduced in multiple sclerosis and restored by low-dose naltrexone. Exp Biol Med 242:1524–1533.

Lueptow LM, Fakira AK, Bobeck EN (2018) The contribution of the descending pain modulatory pathway in opioid tolerance. Front Neurosci 12:886.

Luger NM, Sabino MAC, Schwei MJ, Mach DB, Pomonis JD, Keyser CP, Rathbun M, Clohisy DR, Honore P, Yaksh TL, Mantyh PW (2002) Efficacy of systemic morphine suggests a fundamental difference in the mechanisms that generate bone cancer vs. inflammatory pain. Pain 99:397–406.

Lynch JL, Alley JF, Wellman L, Beitz AJ (2008) Decreased spinal cord opioid receptor mRNA expression and antinociception in a Theiler's murine encephalomyelitis virus model of multiple sclerosis. Brain Res 1191:180–191.

Maarrawi J, Peyron R, Mertens P, Costes N, Magnin M, Sindou M, Laurent B, Garcia-Larrea L (2007) Differential brain opioid receptor availability in central and peripheral neuropathic pain. Pain 127:183–194.

Manning BH (1998) A lateralized deficit in morphine antinociception after unilateral inactivation of the central amygdala. J Neurosci 18:9453–9470.

Manning BH, Mayer DJ (1995) The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. J Neurosci 15:8199–8213.

Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: An in situ hybridization study. J Comp Neurol 350:412–438.

Marchettini P, Formaglio F, Lacerenza M (2006) Pain as heralding symptom in multiple sclerosis. Neurol Sci 27:s294–s296.

Martikainen IK, Peciña M, Love TM, Nuechterlein EB, Cummiford CM, Green CR, Harris RE, Stohler CS, Zubieta JK (2013) Alterations in endogenous opioid functional measures in chronic back pain. J Neurosci 33:14729–14737.

Martin TJ, Kim SA, Buechler NL, Porreca F, Eisenach JC (2007) Opioid self-administration in the nerve-injured rat: Relevance of antiallodynic effects to drug consumption and effects of intrathecal analgesics. Anesthesiology 106:312–322.

McGaraughty S, Heinricher MM (2002) Microinjection of morphine into various amygdaloid nuclei differentially affects nociceptive responsiveness and RVM neuronal activity. Pain

96:153–162.

McMahon SB, Cafferty WBJ, Marchand F (2005) Immune and glial cell factors as pain mediators and modulators. Exp Neurol 192:444–462.

Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994) The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 33:1471–1478.

Metz AE, Yau HJ, Centeno MV, Apkarian AV, Martina M (2009) Morphological and functional reorganization of rat medial prefrontal cortex in neuropathic pain. Proc Natl Acad Sci U S A 106:2423–2428.

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

Milligan ED, Mehmert KK, Hinde JL, Harvey LO, Martin D, Tracey KJ, Maier SF, Watkins LR (2000) Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. Brain Res 861:105–116.

Miyazawa Y, Takahashi Y, Watabe AM, Kato F (2018) Predominant synaptic potentiation and activation in the right central amygdala are independent of bilateral parabrachial activation in the hemilateral trigeminal inflammatory pain model of rats. Mol Pain 14:1–21.

Moehring F, Halder P, Seal RP, Stucky CL (2018) Uncovering the cells and circuits of touch in normal and pathological settings. Neuron 100:349–360.

Mogil JS (2012) Sex differences in pain and pain inhibition: Multiple explanations of a controversial phenomenon. Nat Rev Neurosci 13:859–866.

Mogil JS, Bailey AL (2010) Sex and gender differences in pain and analgesia. Prog Brain Res 186:140–157.

Morgan MM, Fossum EN, Levine CS, Ingram SL (2006) Antinociceptive tolerance revealed by cumulative intracranial microinjections of morphine into the periaqueductal gray in the rat. Pharmacol Biochem Behav 85:214–219.

Moulin DE, Foley KM, Ebers GC (1988) Pain syndromes in multiple sclerosis. Neurology 38:1830–1834.

Mutso AA, Radzicki D, Baliki MN, Huang L, Banisadr G, Centeno M V., Radulovic J, Martina M, Miller RJ, Vania Apkarian A (2012) Abnormalities in hippocampal functioning with persistent pain. J Neurosci 32:5747–5756.

Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M, Nanjo K, Matsuzawa K, Yamazaki M, Suzuki T (2006) Chronic pain induces anxiety with concomitant changes

in opioidergic function in the amygdala. Neuropsychopharmacology 31:739–750.

Nazarian A, Christianson CA, Hua X-Y, Yaksh TL (2008) Dexmedetomidine and ST-91 analgesia in the formalin model is mediated by α2A -adrenoceptors: a mechanism of action distinct from morphine. Br J Pharmacol 155:1117–1126.

Neugebauer V (2015) Amygdala pain mechanisms. Handb Exp Pharmacol 227:261–284.

Neugebauer V, Galhardo V, Maione S, Mackey SC (2009) Forebrain pain mechanisms. Brain Res Rev 60:226–242.

Neugebauer V, Li W (2002) Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J Neurophysiol 87:103–112.

Neugebauer V, Li W (2003) Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. J Neurophysiol 89:716–727.

Neugebauer V, Li W, Bird GC, Bhave G, Gereau IV RW (2003) Synaptic plasticity in the amygdala in a model of arthritic pain: Differential roles of metabotropic glutamate receptors 1 and 5. J Neurosci 23:52–63.

Neugebauer V, Li W, Bird GC, Han JS (2004) The amygdala and persistent pain. Neuroscientist 10:221–234.

Niesters M, Dahan A, Kest B, Zacny J, Stijnen T, Aarts L, Sarton E (2010) Do sex differences exist in opioid analgesia? A systematic review and meta-analysis of human experimental and clinical studies. Pain 151:61–68.

Niikura K, Narita M, Narita M, Nakamura A, Okutsu D, Ozeki A, Kurahashi K, Kobayashi Y, Suzuki M, Suzuki T (2008) Direct evidence for the involvement of endogenous β-endorphin in the suppression of the morphine-induced rewarding effect under a neuropathic pain-like state. Neurosci Lett 435:257–262.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314–1318.

Olechowski CJ, Tenorio G, Sauve Y, Kerr BJ (2013) Changes in nociceptive sensitivity and object recognition in experimental autoimmune encephalomyelitis (EAE). Exp Neurol 241:113–121.

Olechowski CJ, Truong JJ, Kerr BJ (2009) Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE). Pain 141:156–164.

Orton SM, Herrera BM, Yee IM, Valdar W, Ramagopalan S V, Sadovnick AD, Ebers GC (2006) Sex ratio of multiple sclerosis in Canada: a longitudinal study. Lancet Neurol 5:932–936.

Österberg A, Boivie J, Thuomas K-Å (2005) Central pain in multiple sclerosis - prevalence and clinical characteristics. Eur J Pain 9:531–531.

Ozaki S, Narita M, Narita M, Iino M, Miyoshi K, Suzuki T (2003) Suppression of the morphine-induced rewarding effect and G-protein activation in the lower midbrain following nerve injury in the mouse: Involvement of G-protein-coupled receptor kinase 2. Neuroscience 116:89–97.

Ozaki S, Narita M, Narita M, Iino M, Sugita J, Matsumura Y, Suzuki T (2002) Suppression of the morphine-induced rewarding effect in the rat with neuropathic pain: implication of the reduction in μ-opioid receptor functions in the ventral tegmental area. J Neurochem 82:1192–1198.

Panerai AE, Monastra G, Manfredi B, Sacerdote P, Radulovic J, Locatelli L (1994) β-Endorphin concentrations in brain areas and peritoneal macrophages in rats susceptible and resistant to experimental allergic encephalomyelitis: A possible relationship between tumor necrosis factor a and opioids in the disease. J Neuroimmunol 51:169–176.

Pardini M, Capello E, Krueger F, Mancardi G, Uccelli A (2013) Reward responsiveness and fatigue in multiple sclerosis. Mult Scler J 19:233–240.

Passamonti L, Cerasa A, Liguori M, Gioia MC, Valentino P, Nisticò R, Quattrone A, Fera F (2009) Neurobiological mechanisms underlying emotional processing in relapsing-remitting multiple sclerosis. Brain 132:3380–3391.

Pavlovic ZW, Bodnar RJ (1998) Opioid supraspinal analgesic synergy between the amygdala and periaqueductal gray in rats. Brain Res 779:158–169.

Pavlovic ZW, Cooper ML, Bodnar RJ (1996) Opioid antagonists in the periaqueductal gray inhibit morphine and β-endorphin analgesia elicited from the amygdala of rats. Brain Res 741:13–26.

Pelvig DP, Pakkenberg H, Stark AK, Pakkenberg B (2008) Neocortical glial cell numbers in human brains. Neurobiol Aging 29:1754–1762.

Peruga I, Hartwig S, Thöne J, Hovemann B, Gold R, Juckel G, Linker RA (2011) Inflammation modulates anxiety in an animal model of multiple sclerosis. Behav Brain Res 220:20–29.

Petraschka M, Li S, Gilbert TL, Westenbroek RE, Bruchas MR, Schreiber S, Lowe J, Low MJ, Pintar JE, Chavkin C (2007) The absence of endogenous β-endorphin selectively blocks phosphorylation and desensitization of mu opioid receptors following partial sciatic nerve ligation. Neuroscience 146:1795–1807.

Pitteri M, Genova H, Lengenfelder J, DeLuca J, Ziccardi S, Rossi V, Calabrese M (2019) Social cognition deficits and the role of amygdala in relapsing remitting multiple sclerosis patients without cognitive impairment. Mult Scler Relat Disord 29:118–123.

Polanczyk MJ, Jones RE, Subramanian S, Afentoulis M, Rich C, Zakroczymski M, Cooke P, Vandenbark AA, Offner H (2004) T lymphocytes do not directly mediate the protective effect of estrogen on experimental autoimmune encephalomyelitis. Am J Pathol 165:2069–2077.

Pollak Y, Orion E, Goshen I, Ovadia H, Yirmiya R (2002) Experimental autoimmune encephalomyelitis-associated behavioral syndrome as a model of 'depression due to multiple sclerosis.' Brain Behav Immun 16:533–543.

Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, Montalban X, O'Connor P, Sandberg-Wollheim M, Thompson AJ, Waubant E, Weinshenker B, Wolinsky JS (2011) Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann Neurol 69:292–302.

Potter LE, Paylor JW, Suh JS, Tenorio G, Caliaperumal J, Colbourne F, Baker G, Winship I, Kerr BJ (2016) Altered excitatory-inhibitory balance within somatosensory cortex is associated with enhanced plasticity and pain sensitivity in a mouse model of multiple sclerosis. J Neuroinflammation 13:1–20.

Price DD, Verne GN, Schwartz JM (2006) Plasticity in brain processing and modulation of pain. Prog Brain Res 157:333–405.

Price JL (2003) Comparative aspects of amygdala connectivity. Ann N Y Acad Sci 985:50–58.

Prus AJ, James JR, Rosecrans JA (2009) Conditioned Place Preference. In: Methods of Behavior Analysis in Neuroscience. 2nd edition. Springer International Publishing.

Raghavendra V, Rutkowski MD, Deleo JA (2002) The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic pain and sham-operated rats. J Neurosci 22:9980–9989.

Raghavendra V, Tanga F, DeLeo JA (2003a) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 306:624–630.

Raghavendra V, Tanga F, Rutkowski MD, DeLeo JA (2003b) Anti-hyperalgesic and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: Mechanistic implications of spinal glia and proinflammatory cytokines. Pain 104:655–664.

Rangachari M, Kuchroo VK (2013) Using EAE to better understand principles of immune function and autoimmune pathology. J Autoimmun 45:31–39.

Ren W, Kiritoshi T, Grégoire S, Ji G, Guerrini R, Calo G, Neugebauer V (2013) Neuropeptide S: a novel regulator of pain-related amygdala plasticity and behaviors. J Neurophysiol 110:1765–1781.

Ren W, Neugebauer V (2010) Pain-related increase of excitatory transmission and decrease of inhibitory transmission in the central nucleus of the amygdala are mediated by mGluR1. Mol Pain 6:1744-8069-6–93.

Ringkamp M, Meyer RA (2008) Physiology of nociceptors. In: The Senses: A Comprehensive Reference, pp 97–114. Elsevier Inc.

Rizvi TA, Ennis M, Behbehani MM, Shipley MT (1991) Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: Topography and reciprocity. J Comp Neurol 303:121–131.

Robinson AP, Harp CT, Noronha A, Miller SD (2014) The experimental autoimmune encephalomyelitis (EAE) model of MS. Utility for understanding disease pathophysiology and treatment. In: Handbook of Clinical Neurology, pp 173–189. Elsevier B.V.

Rodgers RJ (1978) Influence of intra-amygdaloid opiate injections on shock thresholds, tail-flick latencies and open field behaviour in rats. Brain Res 153:211–216.

Rodrigues DH, Sachs D, Teixeira AL (2009) Mechanical hypernociception in experimental autoimmune encephalomyelitis. Arq Neuropsiquiatr 67:78–81.

Rowbotham MC, Twilling L, Davies PS, Reisner L, Taylor K, Mohr D (2003) Oral opioid therapy for chronic peripheral and central neuropathic pain. N Engl J Med 348:1223–1232.

Sah P, Faber ESL, De Armentia ML, Power J (2003) The amygdaloid complex: Anatomy and physiology. Physiol Rev 83:803–834.

Sandkühler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiol Rev 89:707–758.

Sanna MD, Quattrone A, Galeotti N (2017) Silencing of the RNA-binding protein HuR attenuates hyperalgesia and motor disability in experimental autoimmune encephalomyelitis. Neuropharmacology 123:116–125.

Sarton E, Olofsen E, Romberg R, Den Hartigh J, Kest B, Nieuwenhuijs D, Burm A, Teppema L, Dahan A (2000) Sex differences in morphine analgesia: An experimental study in healthy volunteers. Anesthesiology 93:1245–1254.

Sawada A, Niiyama Y, Ataka K, Nagaishi K, Yamakage M, Fujimiya M (2014) Suppression of bone marrow-derived microglia in the amygdale improves anxiety-like behavior induced by chronic partial sciatic nerve ligation in mice. Pain 155:1762–1772.

Sawamura S, Fujinaga M, Kingery WS, Belanger N, Davies MF, Maze M (1999) Opioidergic and adrenergic modulation of formalin-evoked spinal c-fos mRNA expression and nocifensive behavior in the rat. Eur J Pharmacol 379:141–149.

Schrepf A, Harper DE, Harte SE, Wang H, Ichesco E, Hampson JP, Zubieta J-K, Clauw DJ, Harris RE (2016) Endogenous opioidergic dysregulation of pain in fibromyalgia: a PET and fMRI study. Pain 157:2217–2225.

Schubert DSP, Foliart RH (1993) Increased depression in multiple sclerosis patients: A meta-analysis. Psychosomatics 34:124–130.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry 20:11–21.

Seixas D, Palace J, Tracey I (2016) Chronic pain disrupts the reward circuitry in multiple sclerosis. Eur J Neurosci 44:1928–1934.

Sevostianova N, Danysz W, Bespalov AY (2005) Analgesic effects of morphine and loperamide in the rat formalin test: Interactions with NMDA receptor antagonists. Eur J Pharmacol 525:83–90.

Simon EJ (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357–374.

Simons LE, Moulton EA, Linnman C, Carpino E, Becerra L, Borsook D (2014) The human amygdala and pain: Evidence from neuroimaging. Hum Brain Mapp 35:527–538.

Sorge RE et al. (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nat Neurosci 18:1081–1083.

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

Stoffel EC, Ulibarri CM, Folk JE, Rice KC, Craft RM (2005) Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. J Pain 6:261–274.

Stucky CL, Gold MS, Zhang X (2001) Mechanisms of pain. Proc Natl Acad Sci U S A 98:11845–11846.

Sun N, Cassell MD (1993) Intrinsic GABAergic neurons in the rat central extended amygdala. J Comp Neurol 330:381–404.

Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL (2003) Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. J Neurochem 86:1534–1544.

Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA (1999) Acute peripheral inflammation induces moderate glial activation and spinal IL-1β expression that correlates with pain behavior in the rat. Brain Res 829:209–221.

Tanga F., Raghavendra V, DeLeo J. (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int 45:397–407.

Taves S, Berta T, Liu DL, Gan S, Chen G, Kim YH, Van de Ven T, Laufer S, Ji RR (2015) Spinal inhibition of p38 MAP kinase reduces inflammatory and neuropathic pain in male but not female mice: Sex-dependent microglial signaling in the spinal cord. Brain Behav Immun 55:70–81.

Taylor AMW, Castonguay A, Taylor AJ, Murphy NP, Ghogha A, Cook C, Xue L, Olmstead

MC, De Koninck Y, Evans CJ, Cahill CM (2015) Microglia disrupt mesolimbic reward circuitry in chronic pain. J Neurosci 35:8442–8450.

Taylor AMW, Chadwick CI, Mehrabani S, Hrncir H, Arnold AP, Evans CJ (2020) Sex differences in kappa opioid receptor antinociception is influenced by the number of X chromosomes in mouse. J Neurosci Res.

Taylor AMW, Mehrabani S, Liu S, Taylor AJ, Cahill CM (2017) Topography of microglial activation in sensory- and affect-related brain regions in chronic pain. J Neurosci Res 95:1330–1335.

Thompson JM, Neugebauer V (2017) Amygdala plasticity and pain. Pain Res Manag.

Thompson SJ, Pitcher MH, Stone LS, Tarum F, Niu G, Chen X, Kiesewetter DO, Schweinhardt P, Bushnell MC (2018) Chronic neuropathic pain reduces opioid receptor availability with associated anhedonia in rat. Pain 159:1856–1866.

Tillisch K, Mayer EA, Labus JS (2011) Quantitative meta-analysis identifies brain regions activated during rectal distension in irritable bowel syndrome. Gastroenterology 140:91–100.

Tomassini V, Pozzilli C (2006) Sex hormones: A role in the control of multiple sclerosis? Expert Opin Pharmacother 7:857–868.

Tortorici V, Morgan MM, Vanegas H (2001) Tolerance to repeated microinjection of morphine into the periaqueductal gray is associated with changes in the behavior of off- and on-cells in the rostral ventromedial medulla of rats. Pain 89:237–244.

Totsch SK, Sorge RE (2017) Immune system involvement in specific pain conditions. Mol Pain 13:1–17.

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. J Neurosci 28:4949–4956.

Tracey I, Bushnell MC (2009) How neuroimaging studies have challenged us to rethink: Is chronic pain a disease? J Pain 10:1113–1120.

Trang T, Beggs S, Wan X, Salter MW (2009) P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. J Neurosci 29:3518–3528.

Traub RJ, Silva E, Gebhart GF, Solodkin A (1996) Noxious colorectal distention induced-c-Fos protein in limbic brain structures in the rat. Neurosci Lett 215:165–168.

Treede R-D et al. (2015) A classification of chronic pain for ICD-11. Pain 156:1003–1007.

Tsuda M, Kuboyama K, Inoue T, Nagata K, Tozaki-Saitoh H, Inoue K (2009) Behavioral

phenotypes of mice lacking purinergic P2X4 receptors in acute and chronic pain assays. Mol Pain 5:1744–8069.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778–783.

Vachon-Presseau E, Tétreault P, Petre B, Huang L, Berger SE, Torbey S, Baria AT, Mansour AR, Hashmi JA, Griffith JW, Comasco E, Schnitzer TJ, Baliki MN, Apkarian AV (2016) Corticolimbic anatomical characteristics predetermine risk for chronic pain. Brain 139:1958–1970.

Veinante P, Yalcin I, Barrot M (2013) The amygdala between sensation and affect: a role in pain. J Mol Psychiatry 1.

Voskuhl RR, Gold SM (2012) Sex-related factors in multiple sclerosis susceptibility and progression. Nat Rev Neurol 8:255–263.

Wagner KJ, Valet M, Kochs EF, Kriner M, Tölle TR, Sprenger T (2010) The μ-opioid receptor agonist remifentanil induces acute dysphoria irrespective of its analgesic properties. J Psychopharmacol 24:355–361.

Walter L, Neumann H (2009) Role of microglia in neuronal degeneration and regeneration. Semin Immunopathol 31:513–525.

Wang D, Tawfik VL, Corder G, Low SA, François A, Basbaum AI, Scherrer G (2018) Functional Divergence of Delta and Mu Opioid Receptor Organization in CNS Pain Circuits. Neuron 98:90–108.

Wardle MC, Fitzgerald DA, Angstadt M, Rabinak CA, De Wit H, Luan Phan K (2014) Effects of oxycodone on brain responses to emotional images. Psychopharmacology (Berl) 231:4403–4415.

Wassum KM, Izquierdo A (2015) The basolateral amygdala in reward learning and addiction. Neurosci Biobehav Rev 57:271–283.

Watkins LR, Hutchinson MR, Ledeboer A, Wieseler-Frank J, Milligan ED, Maier SF (2007) Glia as the “bad guys”: Implications for improving clinical pain control and the clinical utility of opioids. Brain Behav Immun 21:131–146.

Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF (1997) Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. Pain 71:225–235.

Wen J, Yablonskiy DA, Salter A, Cross AH (2017) Limbic system damage in MS: MRI assessment and correlations with clinical testing Ginsberg SD, ed. PLoS One 12:e0187915.

Werka T, Marek P (1990) Post-stress analgesia after lesions to the central nucleus of the amygdala in rats. Acta Neurobiol Exp 50:13–22.

Whitacre CC, Reingold SC, O'Looney PA, Blankenhorn E, Brinley F, Collier E, Duquette P, Fox H, Giesser B, Gilmore W, Lahita R, Nelson JL, Reiss C, Riskind P, Voskuhl R (1999) A gender gap in autoimmunity. Science 283:1277–1278.

Willoch F, Schindler F, Wester HJ, Empl M, Straube A, Schwaiger M, Conrad B, Tölle TR (2004) Central poststroke pain and reduced opioid receptor binding within pain processing circuitries: A [11C]diprenorphine PET study. Pain 108:213–220.

Willoch F, Tölle TR, Wester HJ, Munz F, Petzold A, Schwaiger M, Conrad B, Bartenstein P (1999) Central pain after pontine infarction is associated with changes in opioid receptor binding: A PET study with 11C-diprenorphine. Am J Neuroradiol 20:686–690.

Wilson TD, Valdivia S, Khan A, Ahn HS, Adke AP, Gonzalez SM, Sugimura YK, Carrasquillo Y (2019) Dual and opposing functions of the central amygdala in the modulation of pain. Cell Rep 29:332–346.

Winters BL, Gregoriou GC, Kissiwaa SA, Wells OA, Medagoda DI, Hermes SM, Burford NT, Alt A, Aicher SA, Bagley EE (2017) Endogenous opioids regulate moment-to-moment neuronal communication and excitability. Nat Commun 8:1–15.

Woolf CJ (2011) Central sensitization: Implications for the diagnosis and treatment of pain. Pain 152:S2–S15.

Yang Y, Li H, Li TT, Luo H, Gu XY, Lü N, Ji RR, Zhang YQ (2015) Delayed activation of spinal microglia contributes to the maintenance of bone cancer pain in female Wistar rats via P2X7 receptor and IL-18. J Neurosci 35:7950–7963.

Yekkirala AS, Roberson DP, Bean BP, Woolf CJ (2017) Breaking barriers to novel analgesic drug development. Nat Rev Drug Discov 16:545–565.

Yousuf MS, Zubkow K, Tenorio G, Kerr B (2017) The chloride co-transporters, NKCC1 and KCC2, in experimental autoimmune encephalomyelitis (EAE). Neuroscience 344:178–186.

Yuan S, Shi Y, Tang SJ (2012) Wnt signaling in the pathogenesis of multiple sclerosis-associated chronic pain. J Neuroimmune Pharmacol 7:904–913.

Zacny JP, Lichtor SA (2008) Within-subject comparison of the psychopharmacological profiles of oral oxycodone and oral morphine in non-drug-abusing volunteers. Psychopharmacology (Berl) 196:105–116.

Zagon IS, Rahn KA, Turel AP, McLaughlin PJ (2009) Endogenous opioids regulate expression of experimental autoimmune encephalomyelitis: A new paradigm for the treatment of multiple sclerosis. Exp Biol Med 234:1383–1392.

Zanier ER, Fumagalli S, Perego C, Pischiutta F, De Simoni M-G (2015) Shape descriptors of the "never resting" microglia in three different acute brain injury models in mice. Intensive Care Med Exp 3:1–18.

Zhang J, Koninck Y (2006) Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. J Neurochem 97:772–783.

Zhang R-X, Liu B, Wang L, Ren K, Qiao J-T, Berman BM, Lao L (2005) Spinal glial activation in a new rat model of bone cancer pain produced by prostate cancer cell inoculation of the tibia. Pain 118:125–136.

Zhong Y, Zhou L-J, Ren W-J, Xin W-J, Li Y-Y, Zhang T, Liu X-G (2010) The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: The role of tumor necrosis factor-α. Brain Behav Immun 24:874–880.

Zhou F, Zhuang Y, Wang L, Zhang Y, Wu L, Zeng X, Gong H (2015) Disconnection of the hippocampus and amygdala associated with lesion load in relapsing-remitting multiple sclerosis: A structural and functional connectivity study. Neuropsychiatr Dis Treat 11:1749–1765.

Zubieta J-K, Bueller JA, Jackson LR, Scott DJ, Xu Y, Koeppe RA, Nichols TE, Stohler CS (2005) Placebo effects mediated by endogenous opioid activity on mu-opioid receptors. J Neurosci 25:7754–7762.

Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM, Meyer CR, Koeppe RA, Stohler CS (2001) Regional Mu opioid receptor regulation of sensory and affective dimensions of pain. Science (80-) 293:311–315.

Zurek JR, Nadeson R, Goodchild CS (2001) Spinal and supraspinal components of opioid antinociception in streptozotocin induced diabetic neuropathy in rats. Pain 90:57–63.