Chronic Pain in a Mouse Model of Multiple Sclerosis: Contributions of the Kappa Opioid System

by

Caylin Isabelle Chadwick

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Abstract

The perception of pain is a multimodal experience that is encoded by regions of the central nervous system (CNS) that are involved in sensation, affect, and cognition. Chronic pain is not merely the persistence of pain, but a debilitating condition that is associated with neurochemical dysregulation in these regions. In the general population, chronic pain affects approximately 1 in 5 Canadians, but people living with multiple sclerosis (MS) experience chronic pain at a 3x higher rate. Despite this extremely high prevalence, our understanding of the root cause of chronic pain in MS is very limited. In the general chronic pain field, the kappa opioid system is emerging as an important regulator of the CNS regions involved in the sensory, affective, and cognitive components of pain. The goal of this thesis was to evaluate whether changes in the kappa opioid system occur in these regions in a mouse model of MS by probing the *in vivo* function of exogenous kappa opioid receptor (KOR) agonism and by analyzing post-mortem CNS tissue for mRNA and protein expression of the KOR and its endogenous ligand, dynorphin.

To test this, I induced male and female C57Bl/6 mice with experimental autoimmune encephalomyelitis (EAE), an established mouse model of MS. This model results in progressive ascending motor impairment 1-2 weeks post-induction, with symptoms of pain hypersensitivity beginning a few days prior. KOR agonism has recently been implicated in remyelination, but I was able to confirm that daily KOR agonism did not alter EAE severity and therefore did not confound the behavioural results. At onset of motor symptoms (characterized by a weak tail), EAE caused mechanical allodynia, characterized by a lowered paw withdrawal threshold. Systemic KOR agonism (1.6-30 mg/kg, i.p.) produced robust analgesia in both control and EAE mice. However, direct intrathecal injection of a KOR agonist (10 ug) failed to produce analgesia in EAE mice. I

also showed that KOR-induced aversion (10mg/kg, i.p.) was blunted in EAE mice. Following behavioural assessment, I measured KOR and dynorphin mRNA and protein expression in the following CNS regions involved in the three aspects of pain: the spinal cord (sensory), nucleus accumbens (NAc; affective), amygdala (affective), and claustrum (cognitive). I found that KOR mRNA and protein expression was downregulated in the spinal cord of EAE mice, KOR protein expression was upregulated in the NAc of EAE mice, and the mRNA of dynorphin's precursor was downregulated in the claustrum of EAE mice.

Overall, this thesis has confirmed that the kappa opioid system is dysregulated in a mouse model of MS within CNS regions associated with the sensory, affective, and cognitive components of pain. These finding are in line with current chronic pain literature and highlight the importance of bridging the literature gap between the kappa opioid system, chronic pain, and MS. Future research should focus on investigating the nature of the relationship between kappa opioid system dysregulation and MS-induced chronic pain. Knowing whether this dysregulation is a symptom or a cause of MS-induced chronic pain will guide future development of pain therapeutics for people living with MS.

Preface

This thesis is an original work by Caylin I. Chadwick. This research project received approval from the University of Alberta Research Ethics Board and was conducted under the supervision of Drs. Taylor and Kerr, as well as the guidance of Drs. Webber and Kar. All experiments involving laboratory animals were in compliance with the Canadian Council on Animal Care Guidelines and followed 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 the Canadian Institutes of Health Research (Canada Graduate Scholarship – Master's) and from Alberta Innovates (Graduate Studentship in Health Innovation).

To my younger self.

We did it.

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List of Abbreviations

ANOVA	Analysis of variance
BBB	Blood-brain barrier
BLA	Basolateral nucleus of the amygdala
BSA	Bovine serum albumin
CCD	Charge-coupled device
CeA	Central nucleus of the amygdala
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CPA	Conditioned place aversion
CRF	Corticotropin-releasing factor
DAPI	4',6-diamidino-2-phenylindole
DDH	Deep dorsal horn (of the spinal cord)
DOR	Delta opioid receptor
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent *in situ* hybridization
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
i.t.	Intrathecal
Iba1	Ionized calcium-binding adapter molecule-1
IgG	Immunoglobulin G
KOR	Kappa opioid receptor
mg/kg	Milligram (drug) per kilogram (body weight)
MOG	Myelin oligodendrocyte glycoprotein

MOR	Mu opioid receptor
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSNs	Medium spiny neurons
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcSh	Nucleus accumbens shell
NDS	Normal donkey serum
NOR	Nociceptin/orphanin FQ opioid receptor
OPCs	Oligodendrocyte precursor cells
Oprk1	Gene encoding KOR
PVDF	Polyvinylidene difluoride
PBS	Phosphate buffered saline
Pdyn	Gene encoding pre-prodynorphin
PFA	Paraformaldehyde
SDH	Superficial dorsal horn (of the spinal cord)
SEM	Standard error of the mean
SNL	Spinal nerve ligation
U50	U50,488H
VTA	Ventral tegmental area
%MPE	Percent maximum possible effect
50% PRT	Fifty percent positive response threshold

CHAPTER 1: Introduction

1.1 General Overview

Chronic pain is a highly unpleasant and often debilitating condition that affects approximately 1 in 5 Canadians over the age of 18 (Schopflocher et al., 2011). Chronic pain significantly reduces a person's quality of life and is often accompanied by depression, anxiety, fatigue, reduced productivity, impaired ability to complete daily tasks, and job loss (Campbell et al., 2019; Dueñas et al., 2016; Mokdad et al., 2018; Nicholson & Verma, 2004). In 2019, chronic pain was recognized by the Government of Canada as a significant health issue and the Canadian Pain Task Force was formed to make recommendations for improving the lives of those living with chronic pain (Campbell et al., 2019). Chronic pain that presents with neuropathic pain-like symptoms affects up to 10% of the general population (Moore et al., 2014; Van Hecke et al., 2014), which lines up with a telephone-based study in Alberta that found that half of their respondents that live with chronic pain experience symptoms of neuropathic pain (Toth et al., 2009).

The third leading cause of chronic central neuropathic pain in Canada is multiple sclerosis (MS; Scholz et al., 2019). MS is an autoimmune disease that affects 1 in 385 Canadians (Widdifield et al., 2015). This is already one of the highest rates in the world, but Alberta taken separately ranks in at 1 in every 294 people (calculated from reports by Beck et al., 2005). Approximately 60% of people living with MS suffer from chronic pain (Foley et al., 2013), meaning that a person with MS is three times more likely to experience chronic pain that the average Canadian. Despite this high prevalence, only 24% of people with MS-induced chronic pain receive specific, long-lasting treatment for their pain (Ferraro et al., 2018). Furthermore, typical pharmacological pain interventions, including conventional opioids like morphine, may not sufficiently reduce pain in this population (Kalman et al., 2002; Murphy et al., 2017). Overall,

chronic pain is a significant problem, especially the poor management of MS-induced chronic pain.

The lack of effective treatment for chronic pain in people living with MS may be attributable to the distinction between acute and chronic pain. The perception of pain is a multimodal experience that integrates sensory, affective, and cognitive information. When pain becomes chronic, there is not merely the persistence of pain over a prolonged period of time; there are neurological alterations that cause dysfunction within the sensory, affective, and cognitive circuitry that encode pain (Baliki et al., 2012; Bushnell et al., 2013; Jia & Yu, 2017; Tanimoto et al., 2003; Taylor, 2018). Analgesic medications that only target the sensory system, such as aspirin and other non-steroidal anti-inflammatory drugs, are not even recommended as third line therapeutics for chronic pain (Dworkin et al., 2012; Finnerup et al., 2015), suggesting that the affective and cognitive components of pain must also be addressed in order to mitigate chronic pain. The endogenous opioid system is known to modulate all three aspects of pain, and disruptions to the endogenous opioid system in these circuits have been seen in several models of chronic pain (Narita et al., 2006; Obara et al., 2009; Wawrzczak-Bargieła et al., 2020). These alterations can lead to changes in how an individual perceives pain, such as how much force is needed to elicit pain (sensory disturbances), how much suffering the pain causes (affective disturbances), and how difficult it is to shift attention away from the pain (cognitive disturbances). The goal of this thesis was to describe how an animal model of MS changes one branch of the endogenous opioid system (the kappa opioid system) in sensory, affective, and cognitive regions of the central nervous system (CNS) associated with pain.

1.2 Literature Review

1.2.1 Overview of the Kappa Opioid System

The endogenous opioid system is a family of different receptors, ligands, and precursor peptides that were first described as being responsible for endogenous pain attenuation. The receptors are: the kappa opioid receptor (KOR); the mu opioid receptor (MOR); the delta opioid receptor (DOR); and, the more recently discovered nociceptin/orphanin FQ receptor (NOR) (Stevens, 2009). All of these receptors are seven transmembrane $G_{i/o}$ protein-coupled receptors, which means that activation of these receptors results in a reduction in cellular excitability (Al-Hasani & Bruchas, 2011). Despite this similarity in cellular function, the opioid receptors have notable differences in how they impact behaviour. These differences are driven by two factors. Firstly, these receptors have different selectivity for the endogenous opioid ligands. The KOR is selectively activated by dynorphins, which are ligands differentially spliced from the precursor pre-prodynorphin (Kieffer & Gavériaux-Ruff, 2002). The MOR and DOR can both be activated by endorphins and enkephalins, which are ligands differentially spliced from pro-opiomelanocortin and pre-proenkephalin, respectively (Akil et al., 1984). NOR is activated by nociceptin/orphanin FQ, which is a ligand spliced from pre-pronociceptin (Kieffer & Gavériaux-Ruff, 2002). The second factor contributing to differences in behavioural output is receptor distribution. The opioid receptors are found on distinct cellular populations in different regions of the peripheral and central nervous systems (Mansour et al., 1994; Valentino & Volkow, 2018). While the mu opioid system has been the focus of the majority of opioid-related research, the relatively under-researched kappa opioid system is well-situated within the CNS to regulate the sensory, affective, and cognitive components of pain.

1.2.2 The Kappa Opioid System in the Spinal Cord

The first region of the CNS in which the kappa opioid system is primed to mediate pain – specifically the sensory component – is the spinal cord. The gray matter of the spinal cord can be broken down into the dorsal and ventral horns, which house sensory and motor nerves, respectively. The dorsal horn of the spinal cord can be further delineated into the superficial dorsal horn (SDH; Rexed laminae I and II) and the deep dorsal horn (DDH; Rexed laminae III-V). The SDH contains the central terminals of primary afferents that sense noxious information, while the DDH contains the central terminals of primary afferents that sense innocuous and proprioceptive information (Caspary & Anderson, 2003; Harding et al., 2020). The noxious stimuli-sensing fibres (i.e., nociceptors) synapse onto projection neurons in the SDH, which then relay the nociceptive signal to the thalamus and on to the cortex where it is perceived as painful. This transmission of nociceptive signaling from primary afferents to the brain can be prevented by GABAergic interneurons within the dorsal horn, descending pain inhibitory signaling that terminates in the dorsal horn, or by activating receptors that inhibit the primary or secondary afferents, such as the KOR.

The KOR is expressed throughout the spinal cord, with the highest expression occurring in the SDH (Maekawa et al., 1994; Mansour et al., 1996). In this region, the KOR can be found on the afferent terminals of a subset of peptidergic nociceptors that express high levels of the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Snyder et al., 2018). Activation of these presynaptic KORs hyperpolarizes the membrane and decreases the likelihood of nociceptive signal propagation, leading to the antinociception achieved by intrathecal KOR agonism (Fleetwood-Walker et al., 1988; Han et al., 1984; Millan et al., 1989; Pelissier et al., 1990;

Wood et al., 1981). The KOR is also expressed on the projection neurons in the SDH, providing a postsynaptic antinociceptive mechanism (Besse et al., 1990; Randić et al., 1995). Additionally, the KOR can be found on the afferent terminals of a subset of low threshold mechanoreceptors and GABAergic interneurons in the DDH (Snyder et al., 2018; Xu et al., 2004), but both populations are likely to be more involved in itch perception than pain (S. Chen et al., 2020; Munanairi et al., 2018; Sakai et al., 2020). Overall, the kappa opioid system is poised to be an important regulator of pain transmission in the spinal cord.

When pain becomes chronic, many of the body's functions can become dysregulated, including KOR-mediated spinal antinociception. The first change of note is that dynorphin protein and/or pre-prodynorphin mRNA expression increases in models of persistent hindpaw inflammation (Millan et al., 1988; R. L. Nahin et al., 1989; Ruda et al., 1988), arthritis-induced chronic pain (Millan et al., 1986), spinal cord injury (Faden et al., 1985; Przewłocki et al., 1988), and neuropathic pain (Wagner et al., 1993). Similarly, increased levels of immunoreactive phosphorylated (i.e., activated) KOR has been reported in a model of neuropathic pain (Xu et al., 2004). There is also evidence that persistent hindpaw inflammation elicits circuitry-level plasticity in dynorphin-expressing neurons of the SDH (Nahin et al., 1992). The most intriguing finding regarding the kappa opioid system in chronic pain is that dynorphin seemingly contributes to the maintenance of chronic neuropathic pain (Z. Wang et al., 2001). In this study, wildtype mice with spinal nerve ligation (SNL) developed mechanical allodynia and thermal hyperalgesia that persisted past two weeks post-SNL. However, homozygous prodynorphin knockout mice with SNL showed recovery of these symptoms just after a week post-SNL. Furthermore, at two weeks post-SNL, wildtype mice treated with intrathecal dynorphin antiserum showed pain thresholds

indistinguishable from baseline (Z. Wang et al., 2001). Interestingly, this same pronociceptive effect of KOR activation was seen when selective KOR agonist U50,488H (U50) was exogenously applied to the spinal cord (Hylden et al., 1991). However, this study showed that only an extremely low dose (197 nmol) was able to facilitate cellular excitation, while larger doses (560 nmol and 1.9 umol) produced the cellular inhibition that is associated with antinociception (Hylden et al., 1991). This dose-dependent contradictory role of spinal KOR agonism highlights the fact that our understanding of the kappa opioid system in spinal antinociception and chronic pain is still evolving and requires more investigation. However, just as the sensory aspect does not fully encompass pain, the spinal cord does not fully encompass the kappa opioid system's contribution to analgesia and chronic pain.

1.2.3 The Kappa Opioid System in the Nucleus Accumbens

The second region of the CNS that my project concerns is the nucleus accumbens (NAc). This brain region is a component of the limbic system, and is often divided into the core (NAcC) and shell (NAcSh) regions. The main inputs are dopaminergic projections from the ventral tegmental area (VTA), and activation of these neurons are heavily implicated in the perceived valence of a stimulus (Berridge & Kringelbach, 2013). The main outputs are medium spiny neurons (MSNs; i.e., GABAergic projection neurons), that have either G_s protein-coupled (D1) or G_i protein-coupled (D2) dopamine receptors (Salgado & Kaplitt, 2015). Traditionally, activation of D1-expressing NAc MSNs has been associated with reward-like behaviours, while activation of D2-expression NAc MSNs has been associated with aversion-like behaviours (Cahill et al., 2014). However, mounting evidence now points to both MSN populations being able to produce positive and negative valence-associated behaviours (Soares-Cunha et al., 2020). The NAc is

classically known to be involved in reward and motivation, but has been recently recognized for its role in pain, particularly the affective component. The most compelling evidence for this connection is that silencing the NAc via intracranial injection of lidocaine relieves tactile and cold allodynia in a neuropathic pain model (Chang et al., 2014). This paper also used functional magnetic resonance imaging (fMRI) to assess functional connectivity of the NAc in neuropathic pain. They found that functional connectivity in the NAcC was negatively correlated with tactile allodynia (Chang et al., 2014). In humans, fMRI studies of patients with subacute back pain that was later confirmed to transition into chronic pain had greater functional connectivity in the NAc than patients whose pain recovered (Baliki et al., 2012). Another study from this group showed that exposure to the same acutely painful stimulus produced different firing patterns only in the NAc of patients with chronic back pain and healthy controls (Baliki et al., 2010). This study also showed that different aspects of NAc activity was correlated with the subjective magnitude of the acutely painful stimulus, with reports of the magnitude of ongoing chronic back pain, and with the subsequent response to analgesia (Baliki et al., 2010). This mounting evidence clearly indicates a role for the NAc in the affective component of pain, but the above studies do not offer a mechanism for this phenomenon.

One potential mechanism is the kappa opioid system. The NAc is rich in KOR and dynorphin expression (C. Chen et al., 2020; Crowley & Kash, 2015). The KOR is found on the presynaptic terminals of dopaminergic projection neurons from the VTA. The antagonistic function of KOR on these neurons has been linked to depression-like behaviours, with inhibition of these receptors providing antidepressant-like effects (Lutz & Kieffer, 2013). Activation of D1-expressing MSNs leads to corelease of dynorphin within the NAc (Al-Hasani et al., 2015). This

dynorphin release has been shown to have differential effects on behaviour depending on the subpopulation of neurons inhibited via dynorphin/KOR binding. This is true for the distinction between D1 and D2 MSNs (Tejeda et al., 2017), as well as between neurons located in the dorsal and ventral NAcSh (Al-Hasani et al., 2015). In terms of pain, increases in KOR transcripts within the NAc has been implicated in pain-induced negative affect in a model of transient inflammatory pain (Massaly et al., 2019). A similar elevation in levels of KOR transcripts in the NAc is thought to be responsible for the aversive nature of chronic neuropathic pain (Liu et al., 2019). In summary, the NAc is known to be involved in the affective component of pain, possibly due to kappa opioid signaling in this region. However, this is not the only region associated with the affective aspect of pain.

1.2.4 The Kappa Opioid System in the Amygdala

The amygdala is another limbic region that has been shown to be highly involved in pain. It is considered one of the main hubs of the pain salience network (for review see: Neugebauer et al., 2004). The amygdala consists of two main nuclei: the basolateral nucleus of the amygdala (BLA) and the central nucleus of the amygdala (CeA). In general, the BLA receives inputs from sensory and cortical regions and then relays that information to the CeA via short-range excitatory neurons. The CeA houses a few subpopulations of GABAergic neurons that serve as the main outputs of the amygdala. The BLA also sends signals directly to the NAc, the claustrum, and the cortex (Neugebauer et al., 2004; Phillips et al., 2003; Smith et al., 2020). The CeA has been found to be particularly important in affective pain processing, with the lateral capsular division of the CeA sometimes referred to as the "nociceptive amygdala" (Neugebauer et al., 2004). In addition to inputs from the BLA, the CeA also receives nociceptive input via the spino-parabrachio-

amygdaloid tract, circumventing the BLA (Bernard & Besson, 1990; Burstein & Potrebic, 1993). One of the populations of GABAergic CeA neurons expresses corticotropin-releasing factor (CRF), which can initiate the hypothalamic-pituitary-adrenal axis, which is known to elicit stress-induced analgesia. Increased activation of CeA neurons has been seen in animal models of chemical and visceral pain (Tanimoto et al., 2003). They found that these noxious stimuli evoked conditioned place aversion to the pain-paired chamber, and that lesioning the CeA – but not BLA – blocked this aversion. Futhermore, neither lesion produced any changes in pain-like behaviours to the stimulus application, which highly suggests a role for the CeA in the affective, but not sensory, component of pain (Tanimoto et al., 2003). Additionally, both the CeA and BLA have been shown to be important in nociceptive and depression-like behaviours in a model of neuropathic pain (Seno et al., 2018). Unsurprisingly, we may once again turn to the kappa opioid system for an explanation of this role of the amygdala in pain.

Although the function of the kappa opioid system has been reliably shown in the amygdala, the specific neuronal mechanisms are still not well defined. We know that the KOR is found on the CeA terminals of excitatory BLA projection neurons, and dynorphin mRNA can be found in the CRF-expressing CeA neurons (Neugebauer et al., 2020). However, the presence of the kappa opioid system in these subpopulations does not fully account for the behavioural effects seen of direct amygdalar administration of a KOR agonist. Both in the CeA and the BLA, agonism of the KOR produces anxiety-like and pronociceptive behaviours that are associated with increased CRF signaling (Bruchas et al., 2009; Hein et al., 2021; Ji & Neugebauer, 2020). This lines up nicely with the increased amygdalar KOR signaling and negative affective behaviours in models of neuropathic pain (Narita et al., 2006; Navratilova et al., 2019), although this may be a specific

function of the right CeA (Nation et al., 2018; Phelps et al., 2019). Overall, KOR signaling in the amygdala seems to have a pronociceptive effect opposing its antinociceptive effects in the spinal cord. The evidence points to KOR agonism in the amygdala and NAc both contributing to the encoding of the affective component of pain.

1.2.5 The Kappa Opioid System in the Claustrum

The final component of pain is cognitive. One aspect of this is how salient the pain is (i.e., how much attention is allocated to the pain). The claustrum is a largely understudied region that has extremely high connectivity with the rest of the brain, and is hypothesized to integrate limbic and sensory information and mediate the salience of these stimuli through projections to the frontal cortex (Smith et al., 2020). This aids in the speculation that the claustrum plays a significant role in consciousness (Crick & Koch, 2005; Goll et al., 2015). The claustrum is also well situated within the pain salience network and has reciprocal connections with the BLA (Jackson et al., 2020). The claustrum has been shown to increase activity in an animal model of hindpaw inflammation (Słoniewski et al., 1995) and in human fibromyalgia patients who score high in surveys of pain catastrophizing (Gracely et al., 2004). Decreases in claustrum activity have been correlated with both placebo analgesia and pain anticipation (Amanzio et al., 2013; Palermo et al., 2015), implying a role for the claustrum in the cognitive aspect of pain perception. Interestingly, a meta-analysis of clinical trials for migraine showed a decrease in claustrum grey matter volume (Jia & Yu, 2017), and a model of brain injury showed that nociceptive thresholds were negatively correlated with damage to the claustrum (Persinger et al., 1997). This may indicate a dysfunction of claustrum pain circuitry in chronic conditions, similar to other brain regions.

Investigations of the kappa opioid system in the claustrum have been minimal, despite evidence that the claustrum contains the highest density of KORs in the CNS (C. Chen et al., 2020). However, the hallucinogenic effect of a recreationally used KOR agonist, Salvinorin A, is likely due to the high presence of kappa opioid receptors in this region, and supports the role of the claustrum in cognitive processing (Addy et al., 2015; Stiefel et al., 2014). Overall, more investigation is needed to confirm whether the presence of KORs in the claustrum contributes to its role in the cognitive aspect of pain. Subsequent studies will then need to investigate whether changes in claustrum KOR expression occur in models of chronic pain, like they do in the CNS regions associated with the sensory (spinal cord) and affective (NAc and amygdala) aspects of pain.

1.2.6 The Kappa Opioid System in Multiple Sclerosis

Despite the evidence that various models of chronic pain dysregulate the kappa opioid system, this has been predominantly unresearched in a model of MS. As stated earlier, MS is the third most common cause of chronic central neuropathic pain in Canada, however, pain is not its main disease process. MS is a debilitating disease that causes demyelination, inflammation, and autoimmune reactions throughout the CNS. The myelin sheath is a protective layer produced by oligodendrocytes that surrounds a neuron's axon. This layer increases speed of synaptic transmission, protects the axon from neurodegeneration, and secretes factors necessary for neuronal survival (Bankston et al., 2013). The demyelination that occurs in MS leaves the neuron vulnerable to degeneration, which can elicit some of the highly problematic symptoms of the disease, including sensory, motor, and cognitive deficits (Compston & Coles, 2008). These localized events can occur anywhere within the brain and spinal cord, resulting in highly variable

symptom presentation. Despite this variability, approximately 60% of people living with MS experience chronic pain (Foley et al., 2013). This hypersensitivity can be reproduced by a model of MS known as experimental autoimmune encephalomyelitis (EAE; Olechowski et al., 2009, 2013; Thorburn et al., 2016), and is associated with microglial activation within the amygdala (Dworsky-Fried et al., 2021). However, we do not know whether the kappa opioid system is involved in this pain presentation.

Past explorations of the kappa opioid system in MS have been minimal. The only study to look at KOR expression changes found that MOR, KOR, and DOR mRNA were each decreased in the spinal cords of mice induced with an MS model distinct from EAE (Lynch et al., 2008). The only behavioural results they reported that looked specifically at KOR was a positive correlation between spinal KOR mRNA expression and thermal tail withdrawal response times (i.e., the fewer KOR transcripts, the more sensitive to heat). However, identical correlations were found with MOR and DOR transcripts (Lynch et al., 2008). This is the extent of the literature regarding pain and KOR expression in MS.

The remaining studies looking at KOR in MS have all been to do with disease progression and, more specifically, remyelination (Wang & Mei, 2019). Two studies published in 2016 independently identified KOR mRNA in oligodendroglia and showed that KOR activation on oligodendrocyte precursor cells (OPCs) promoted differentiation of OPCs into mature oligodendrocytes and subsequent myelination (Du et al., 2016; Mei et al., 2016). The beneficial effect of KOR agonists on myelination is abolished in mice that have KOR conditionally knocked out in OPCs (Olig2-Cre; KOR fl/fl), providing evidence that KOR ligands are directly acting on

the KORs expressed on OPCs (Mei et al., 2016). Similarly, Du and colleagues (2016) demonstrated that genetic deletion of KOR worsens the disease severity of EAE, whereas daily administration of the selective KOR agonist, U50 (1.6 mg/kg, i.p., beginning at EAE induction), improves the condition through promoting oligodendrocyte differentiation and remyelination (Du et al 2016). In line with these results, treatment with U50 also enhances remyelination in lysolecithin-, hypoxia-, and cuprizone-induced demyelination (Mei et al., 2016; Wang et al., 2018). Despite the known role of the kappa opioid system in pain and analgesia, and the known comorbidity of pain in MS, none of these studies looked at the influence of their therapies on behavioural measures outside of disease severity.

1.3 Research Aims and Hypotheses

This thesis was designed to bridge the gap in the MS literature regarding the kappa opioid system and its role in the sensory, affective, and cognitive aspects of pain. Firstly, I studied the behavioural effects of the selective and blood-brain barrier (BBB)-permeable KOR agonist U50,488H (U50). Specifically, I tested whether daily U50 injection (1.6 mg/kg, i.p.) had any disease-modifying effects when starting at symptom onset rather than prophylactically as the study described above had done (Du et al., 2016). I also used U50 to probe for behaviourally-relevant changes in the endogenous opioid system, such as alterations to KOR-mediated analgesia and aversion. Secondly, I evaluated various regions of the CNS for expression level changes in the kappa opioid system. Specifically, I looked at mRNA levels of KOR (*Oprk1*) and pre-prodynorphin (*Pdyn*) as well as protein levels of KOR in the lumbar spinal cord, the NAc (core and shell), the amygdala (BLA and CeA), and the claustrum. Based on the available literature, I

hypothesized that EAE results in differential expression of the endogenous kappa opioid system throughout the CNS which contributes to altered behavioural responses to KOR agonism.

CHAPTER 2: Methods

2.1 Animals and Ethics

All experimental procedures received ethics approval from Health Sciences Laboratory Animal Services, a branch of the University of Alberta's Animal Care and Use Committee, and were conducted in accordance with the Canadian Council on Animal Care Guidelines. All experiments were conducted on eight to twelve week old male (N=81) and female (N=81) C57Bl/6 mice (Charles River, Canada). Mice were group housed (4-5 mice per cage) in Ehret mouse cages with *ad libitum* access to water and standard food. Experimental procedures were conducted during the light phase of a 12:12 h light/dark cycle. After arrival, mice were allowed a week of no handling to acclimate to the animal housing facility. The following week was spent slowly introducing mice to cage transportation, the testing environment, experimenter handling, and the behavioural apparatuses to minimize the confound of stress in the experimental results. Mice were differentiated using tail markings with a non-toxic permanent marker.

2.2 EAE Model

2.2.1 Induction

Mice (N=73 [35 males, 38 females) were inoculated via subcutaneous injection with 50 ug of a CNS-specific myelin peptide (MOG_{35-55}; Stanford University Peptide Synthesis Facility) emulsified in 100 uL of Complete Freund's Adjuvant (CFA; Sigma-Aldrich). This emulsion was split into two doses concurrently injected over each posterior flank. Each mouse also received 300 ng of pertussis toxin (List Biological Laboratories) intraperitoneally (i.p.) immediately following inoculation and again 48 hours thereafter. The CFA is an inflammatory agent that induced the mounting of an immune response against MOG_{35-55}. The pertussis toxin caused permeabilization of the BBB, which allowed for immune cells that had produced antibodies against the exogenous

myelin to cross into the CNS and begin attacking the endogenous myelin. These mice went on to develop MS-like demyelination, inflammation, and autoimmune reactions, and were termed the "EAE" group. An equal number of vehicle control mice (N=73 [38 males, 35 females]) receive the same CFA and pertussis injections without the presence of MOG_{35-55}. These mice did not go on to mount an autoimmune attack nor develop MS-like symptoms, and were thus termed the "CFA"[-only] group.

2.2.2 Scoring

EAE and CFA mice were monitored for weight loss and clinical signs from three days post-inoculation onward. The first sign of EAE typically presented ten to eighteen days post-inoculation and took the form of a flaccid or paralyzed tail. This was given an EAE score of 1, provided there was no hindlimb involvement, as EAE typically resulted in an ascending pattern of worsening muscle control in the CNS. Any indication of weakness in the hindlimbs – such as wobbly gait, pelvis not being lifted adequately when walking, or impaired righting reflex – resulted in a score of 2. A score of 3 indicated major hindlimb impairment, usually with those limbs dragging behind the body. However, the distinction between a score of 3 and score of 4 is that mice with a score of 3 would still be using their affected paws to help push their body along the ground, while mice with a score of 4 exhibited total hindlimb paralysis. A score of 5 indicated that the mouse was moribund. EAE-induced mice were excluded if they did not show an EAE score above 0 by 28 days post-induction, unless they showed signs of mechanical allodynia or spinal cord inflammation (N=11).

2.3 Behavioural Assays

2.3.1 Mechanical Paw Withdrawal Thresholds

Evoked mechanical paw withdrawal thresholds were measured with von Frey filaments using the classic up-down method (Chaplan et al., 1994). Mice were habituated to the testing apparatus over two days in one hour sessions, and were allowed to acclimate to the apparatus for 20 minutes before each testing session. Each hindpaw was exposed to the 0.4 g filament first, which was held on the paw for a maximum of five seconds in the case that no pain behaviours (such as shaking, attending to, or licking the paw) were elicited. So long as there was no response, filaments of escalating force were applied to the hindpaw in the same manner until a filament produced a pain behaviour. From there, an additional four filaments were tested, with a pain response followed by a weaker filament and a lack of pain response followed by a stronger filament. These binary responses served to calculate the 50% positive response threshold (PRT) – the force needed to elicit a pain response to 50% of exposures (Dixon, 1980). Data were collected from left and right hindpaws (separated by a 10 minute break) and then averaged to produce one 50% PRT per animal for each round of testing. A lowering of this threshold from the individual's baseline would indicate mechanical allodynia.

This assay was performed at onset (i.e., the first presentation of clinical symptoms) to establish whether mechanical allodynia was present in the EAE animals. This timepoint was chosen because animals at onset present with a score of 1, so there was no hindpaw weakness to confound the results. After establishing evoked mechanical pain thresholds, one cohort of mice were immediately injected with U50,488H (U50; Tocris; 1.6 mg/kg, i.p.; N=18 [10 CFA, 8 EAE]), or saline (0.9%, i.p.; N=18[9 CFA, 9 EAE]). Mice were retested 20 minutes later with the

mechanical paw withdrawal assay. After this second round of threshold measurements, a second injection of U50 (30 mg/kg, i.p.; N=13 [8 CFA, 5 EAE]) or saline (0.9%, i.p.; N=13 [8 CFA, 5 EAE]) was given. This was followed by a third threshold assessment 20 minutes later. Increases in 50% PRT from initial results at onset indicated an analgesic effect of U50.

A separate cohort of EAE (N=12) and CFA (N=12) mice were given U50 via intrathecal injection. After establishing evoked mechanical paw withdrawal thresholds at onset, mice were anaesthetized using isoflurane gas. An insulin needle was then inserted into the intrathecal space between L5 and L6 vertebrae, and 10 ug of U50 diluted in 10 uL of 0.9% saline was administered into the cerebrospinal fluid surrounding the cauda equina. Mice were taken off anesthesia immediately upon removal of the needle, and were given 5 minutes to recover before re-evaluating mechanical paw withdrawal thresholds. A small amount of Evans Blue was added to the drug solution to confirm injection site post-mortem. The paw withdrawal thresholds were excluded if the Evans Blue was found outside of the spinal cord (i.e., failed injection; N=5).

2.3.2 Thermal Tail Withdrawal Thresholds

Evoked thermal tail withdrawal thresholds were measured by gently restraining a mouse and immersing their tail in 49°C water. A stopwatch was used to measure the exact amount of time it took for a mouse to withdraw their tail from the water. If no withdrawal attempt was made, the trial was terminated at a maximum of 15 seconds so as to avoid any tissue damage. Each mouse was tested three times with intervening 5 minute breaks and the trials were averaged to calculate an individual's baseline thermal tail withdrawal threshold. This assay was performed on naïve male (N=8) and female (N=8) C57Bl/6 mice (Charles River, Canada) to confirm the analgesic

effects of U50 at varying doses (0.16, 1.6, 4, and 16 mg/kg, i.p. after 20 minutes) and timepoints (1.6 mg/kg, i.p. after 20, 40, 60, 80, 100, and 120 minutes). This assay was chosen over the above assay because the naïve thermal tail withdrawal thresholds were much further from the assay's maximum threshold than naïve mechanical paw withdrawal thresholds, so effects of the drug were not be confounded by a ceiling effect.

2.3.3 Conditioned Place Aversion

EAE (N=10) and CFA (N=9) mice were individually habituated for 30 minutes to an unbiased conditioned place preference/aversion box with two visually distinct chambers of equal size (24 x 24.5 x 28 cm) separated by a removable door. This door remained open during the habituation to allow the mouse equal opportunity to explore each chamber. Mouse activity was continuously recorded by a ceiling-mounted CCD camera connected to a computer running behavioural tracking software (Ethovision). Time spent in each chamber was calculated so as to account for any innate preference a mouse might have had. The mouse then underwent two days of conditioning an injection of U50 (10 mg/kg, i.p.) with chamber X and an injection of saline (0.9%, i.p.) with chamber Y. Each session lasted 30 minutes. On the final day, the mouse was once again given 30 minutes to freely explore between the chambers with the door open and without receiving any injections. If the mouse spent more time in the saline-paired chamber, it indicated an aversion to the drug. If the mouse spent more time in the drug-paired chamber, it indicated a preference of the drug over saline. If the mouse spent equal time in each chamber, it indicated no aversion or preference. This assay was performed on EAE and CFA mice at days 5-8 post-inoculation (i.e., before the onset of clinical signs), so as to remove the confound of reduced locomotor activity due to EAE onset.

2.4 Tissue Analysis

2.4.1 Tissue Extraction and Preparation

Following behavioural testing, mice were euthanized via pentobarbital injection (0.1 mL, i.p. of 340 mg/ml sodium pentobarbital; Euthasol). Tissue was prepared for immunohistochemical analysis in one group of animals (N=19) using a transcardic perfusion of 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1M phosphate buffer. Spinal cords were then extracted and underwent post-fixation in the fresh 4% PFA for 48 hours and 30% sucrose for 24 hours. Tissue was prepared for *in situ* staining in a second group of animals (N=47) using transcardiac perfusion of ice cold saline (0.9% NaCl). Brains and spinal cords were extracted and frozen into cryomolds for sectioning. A third group of animals (N=64) were euthanized and fresh brains and spinal cords were extracted and homogenized for western blotting.

2.4.2 Immunohistochemistry

After post-fixation, lumbar spinal cords were frozen into cryomolds and sliced onto slides in 20 um thick sections. Slides underwent three 10 minute washes in 1x phosphate buffered saline (PBS) at room temperature before a 60 minute incubation in blocking solution – 10% normal donkey serum (NDS) in 1x PBS + 0.1% Triton X 100 (PBS_{TX}). After blocking, the tissue was then incubated overnight at room temperature with a 1:500 dilution of the primary antibody rabbit anti-Iba1 (Wako) in freshly made antibody solution – PBS_{TX} with 2% NDS and 2% bovine serum albumin (BSA). The next day, the slides underwent two 10 minute washes in 1x PBS + 0.1% tween (PBS_{tween}) and a third 10 minute wash in 1x PBS only. Slides were then incubated for 45 minutes at room temperature with a 1:200 dilution of the secondary antibody donkey anti-rabbit Alexa Fluor 488 (Invitrogen-Life Technologies) in the above described antibody solution. Slides were

then washed again with two 10 minute washes in PBS_{tween} and one 10 minute was in 1x PBS. Slides were then mounted using ProLong Gold with DAPI and coverslipped for imaging using an epifluorescent microscope (Zeiss) at 20x magnification. Cell body size of Iba1 positive cells was measured using ImageJ.

2.4.3 Fluorescent *in situ* Hybridization

CNS tissue was frozen into cryomolds and then sliced onto slides in 12 um thick sections. Slides were immersed in 4% PFA in 0.1M phosphate buffer for 15 minutes at 4°C, then underwent a room temperature dehydration consisting of five minutes each in 50%, 70%, and twice 100% ethanol. Slides were allowed to dry before a hydrophobic barrier was drawn around the tissue samples (ImmEdge® Hydrophobic Barrier PAP Pen). Tissue sections were then incubated for 30 minutes with RNAscope Protease III (ACDbio) at room temperature. Following two quick washes in 1x PBS, slides were incubated at 40°C for two hours with RNAscope probes (ACDbio) for *Oprk1* (the transcript encoding KOR) and *Pdyn* (the transcript encoding prodynorphin – the precursor peptide for dynorphin). Slides were then sequentially incubated with amplification reagents 1-4 with intervening washes in RNAscope wash buffer (ACDbio). Slides were then mounted using ProLong Gold with DAPI and coverslipped for imaging using an epifluorescent microscope (Zeiss) at 40x magnification. Analysis was carried out using ImageJ (lumbar spinal cord) or Zeiss Zen (NAc, amygdala, and claustrum) and quantified the number of fluorescent particles.

2.4.4 Western Blotting

Tissue sections were initially homogenized in 200 uL of tissue extraction reagent I (TER; Invitrogen) with protease inhibitor cocktail (Roche cOmplete EDTA-free tablets), centrifuged at 16,000 xg for 10 minutes at 4°C, and the supernatant was collected. Colorimetric dilution analysis was performed using Bio-Rad Detergent Compatible Protein Assay, and all samples were subsequently diluted to 1 ug/uL aliquots in a 4:1 ratio of TER to Bio-Rad loading buffer. Aliquots were heated to 100°C for 10 minutes and cooled to room temperature before being loaded into Bio-Rad 4-20% Mini-PROTEAN TGX Stain-Free Gels and undergoing electrophoresis at 100-120 V for 60-90 minutes. The gel was then activated for 5 minutes in a ChemiDoc Imager, before having the protein be transferred to a Millipore Immobilon-FL Polyvinylidene Difluoride Membrane using a 300 mA current for 60 minutes at 4°C (lumbar spinal cord) or using a Bio-Rad TransBlotTurbo at 25V up to 1.0A for 20 minutes at room temperature (NAc, amygdala, and claustrum). After the transfer, the membrane underwent total protein analysis in the ChemiDoc Imager, which was subsequently used as a lane-dependent loading control (Aldridge et al., 2008; Eaton et al., 2013; Gilda & Gomes, 2013; Zhai et al., 2015). Membranes were then blocked for 60 minutes at room temperature with 10% BSA and 5% milk power in PBS_{tween}. Immediately post-blocking, the membranes were covered in the primary antibody solution – 1:20,000 (or 1:25,000) rabbit anti-OPRK1 (ThermoFisher Scientific) in PBS_{tween} with 1% BSA (antibody buffer) – overnight at 4°C. The next day, the membranes underwent three washes of 10 minutes each (PBS_{tween} twice followed by 1x PBS once), before a 60 minute incubation at room temperature covered in secondary antibody solution (1:100,000 [or 1:50,000] goat anti-rabbit IgG HRP in antibody buffer). After another three identical washes, the membranes were incubated for 60 seconds with a 1:1 ratio of ECL Western Blotting Detection Reagents 1 & 2. Immediately

thereafter, the membranes were imaged in the ChemiDoc Imager to locate the bands for quantification. The program Image Lab was used to analyze each membrane. Each stained protein band was expressed as a ratio to the total protein in that lane as imaged before membrane staining. Results were further normalized within each membrane to the group of CFA mice that did not receive U50. Replicates of each sample were run on separate gels, and each gel contained a representative from each distinct experimental group.

2.5 Statistical Analysis

For each experiment, the data were tested for normality using the D'Agostino Pearson normality test. If normal, the data underwent parametric statistical tests (unpaired two-tailed Student's t test for two groups, and two- or three-way analysis of variance [ANOVA] for more than two groups). If data did not pass normality testing, data were tested for statistical outliers using the ROUT method (Q=1%). If this did not reveal the data to be normal, nonparametric statistical analyses were performed (two-tailed Mann-Whitney test for two groups, and multiple two-tailed Mann-Whitney tests for more than two groups). For single groups of data that needed to be compared to a theoretical mean, a one sample t test was performed. All statistical analyses were performed using GraphPad Prism (version 9.1.1). Data are presented as means +/- the standard error of the mean (SEM), and differences were considered statistically significant when $p<0.05$.

CHAPTER 3: Results

3.1 Establishing the EAE Model

3.1.1 EAE induction results in weight loss, symptom presentation, and mechanical allodynia

Following EAE induction, mice began showing motor symptoms anywhere from one week to three weeks later. Although there were individual variations in timing of onset, the disease progressed in a similar manner across animals. Both male and female EAE mice showed a downward trend in weight as compared to the CFA animals beginning before symptom onset (Figure 1a; three-way ANOVA, time x disease interaction, $F(9,279)=25.25$, $p<0.0001$), with sex also playing a moderating role (three-way ANOVA, time x sex x disease interaction, $F(9,279)=3.585$, $p=0.0003$). Male EAE mice showed a significantly more severe progression than female EAE mice (Figure 1b; two-way ANOVA, time x sex interaction, $F(9,135)=4.318$, $p<0.0001$). EAE also caused robust spinal cord inflammation. At EAE onset, Iba1 immunoreactive cells (microglia and infiltrating macrophages) in the dorsal horn of the spinal cord had significantly larger cell body sizes in EAE compared to CFA animals (Figures 1c-d; unpaired t test, $t(17)=5.060$, $p<0.0001$). There were no sex differences in Iba1-positive cell body sizes of CFA or EAE mice (Figure 1d, males in blue, females in pink; two-way ANOVA, sex, $F(1,15)=0.04201$, $p=0.8404$). This increase in cell body size indicated a more activated phenotype. Also at onset of disease, EAE mice presented with significantly lower mechanical paw withdrawal thresholds than CFA controls (Figure 1e; Mann-Whitney, $U=62$, $p=0.0043$). There was no contribution of sex to these paw withdrawal thresholds (Figure 1e, males in blue, females in pink; multiple Mann-Whitney: CFA, $U=28$, $p=0.1752$; EAE, $U=28$, $p=0.4649$). Overall, the EAE model was confirmed to produce weight loss, motor deficits, spinal inflammation, and mechanical allodynia.

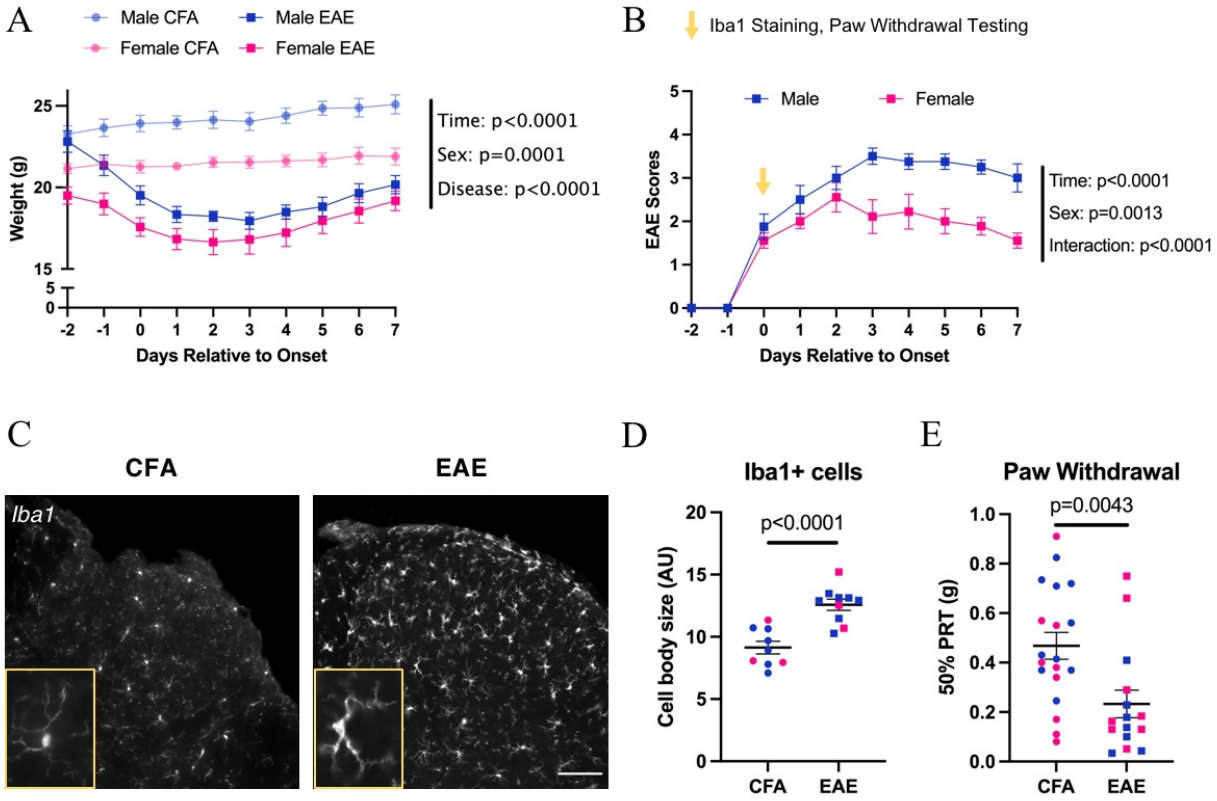

Figure 1: EAE induction results in weight loss, symptom presentation, and mechanical allodynia. (A) Weight tracking of EAE and CFA mice in the days before and after disease onset. EAE prompted a reduction in weight in both males and females. (B) EAE scoring in males and females relative to onset. Males exhibited significantly more severe motor impairment compared to females, particularly in the later timepoints. (C-D) Immunohistochemical staining for Iba1+ microglial cells in the dorsal horn of the spinal cord at disease onset (yellow arrow in B). EAE (12.57 +/- 0.455) significantly increases microglial cell body size compared to CFA (9.137 +/- 0.507). No significant differences between males and females were observed, so data are presented as pooled (males in blue, females in pink). (E) Mechanical paw withdrawal threshold testing at disease onset (yellow arrow in B). 50% positive response threshold (PRT) is significantly lower in EAE mice (0.2327 +/- 0.0557) than CFA mice (0.4679 +/- 0.0544). No significant differences were detected between males and females. Data presented as mean +/- SEM. Scale bar = 100 um. For full statistical details, see Appendix B.

3.1.2 EAE disease progression is unchanged by daily KOR agonism

In order to assess the effects of KOR agonism in the EAE model, I had to first determine whether daily KOR agonism had any effect on disease progression. As mentioned in section 1.2.6, giving U50 (1.6 mg/kg, i.p.) daily beginning at induction lessened disease severity (Du et al.,

2016). Rather than beginning treatment prophylactically, I administered U50 (1.6 mg/kg, i.p.) daily beginning at onset of motor symptoms (Figure 2a). This delay in therapeutic window prevented KOR agonism from having any effect on disease progression in both males (Figure 2b; two-way ANOVA, treatment x time interaction, $F(9,54)=0.1733$, $p=0.9960$) and females (Figure 2c; two-way ANOVA, treatment x time interaction, $F(9,63)=0.4700$, $p=0.8893$). Although daily U50 treatment did not attenuate disease progression, this result does tell us that any change in behavioural effects of KOR agonism in EAE mice is not confounded by an altered disease progression.

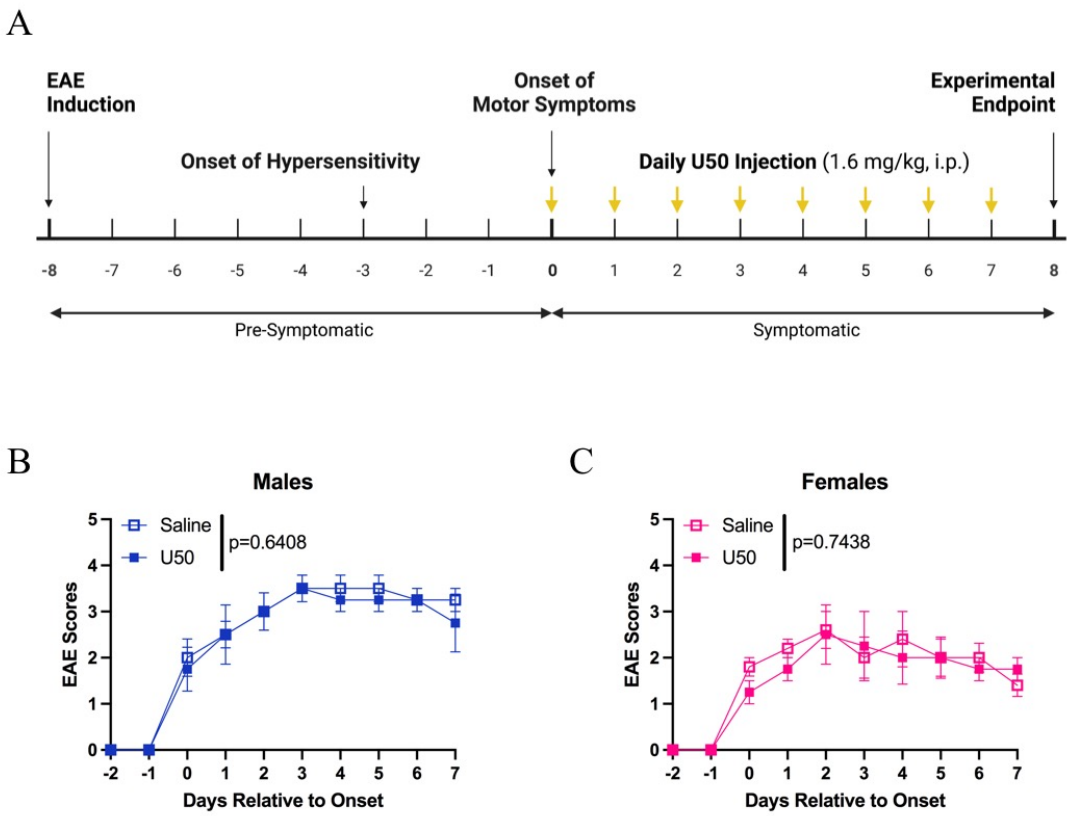

Figure 2: EAE disease progression is unchanged by daily KOR agonism. (A) Experimental timeline. Yellow arrows indicate injection with U50 (1.6 mg/kg, i.p.). (B-C) Progression of EAE scores relative to onset. Neither males (B) nor females (C) showed any difference in EAE disease progression when given daily U50 injections compared to daily saline injections. Data presented as means +/- SEM. For full statistical details, see Appendix B.

3.2 Behavioural Effects of *in vivo* KOR agonism

3.2.1 Systemic KOR agonism is analgesic in naïve mice

The analgesic capacity of U50 was tested in naïve male and female mice using the thermal tail withdrawal assay. U50 given intraperitoneally significantly increased tail flick latency with increasing doses 20 minutes after injection (Figure 3a; two-way ANOVA, dose, $F(2.696,35.04)=23.43$, $p<0.0001$). Sex did not significantly alter these results (Figure 3a; two-way ANOVA, dose x sex interaction, $F(4,52)=0.6922$, $p=0.6007$). When given a single dose of 1.6 mg/kg U50 (i.p.), the analgesic effect increased for 40 minutes (Figure 3b; two-way ANOVA, time, $F(3.863,54.09)=5.560$, $p=0.0009$). However, females showed significantly lower tail flick latencies than males (Figure 3b; two-way ANOVA, sex, $F(1,14)=9.804$, $p=0.0074$). When looking specifically at 1.6 mg/kg U50, tail flick latency significantly increased at 20 minutes post-injection (Figure 3c; two-way ANOVA, drug, $F(1,14)=21.36$, $p=0.0004$). Sex did not alter these results (Figure 3c; two-way ANOVA, sex, $F(1,14)=3.059$, $p=0.1022$). This held true when looking at this data as percent maximum possible effect (%MPE). Both males and females showed a significant increase 20 minutes after 1.6 mg/kg U50 (Figure 3d; one sample t test, theoretical mean: 0; males: $t(7)=3.846$, $p=0.0063$; females: $t(7)=2.656$). There was no significant difference in %MPE between males and females (Figure 3d; unpaired t test, $t(14)=0.7691$, $p=0.4546$). Since 1.6 mg/kg U50 (i.p.) was used for the daily injections tested above, and because these data have shown it to provide antinociception at 20 minutes, this dose and timing was carried forward into subsequent experiments.

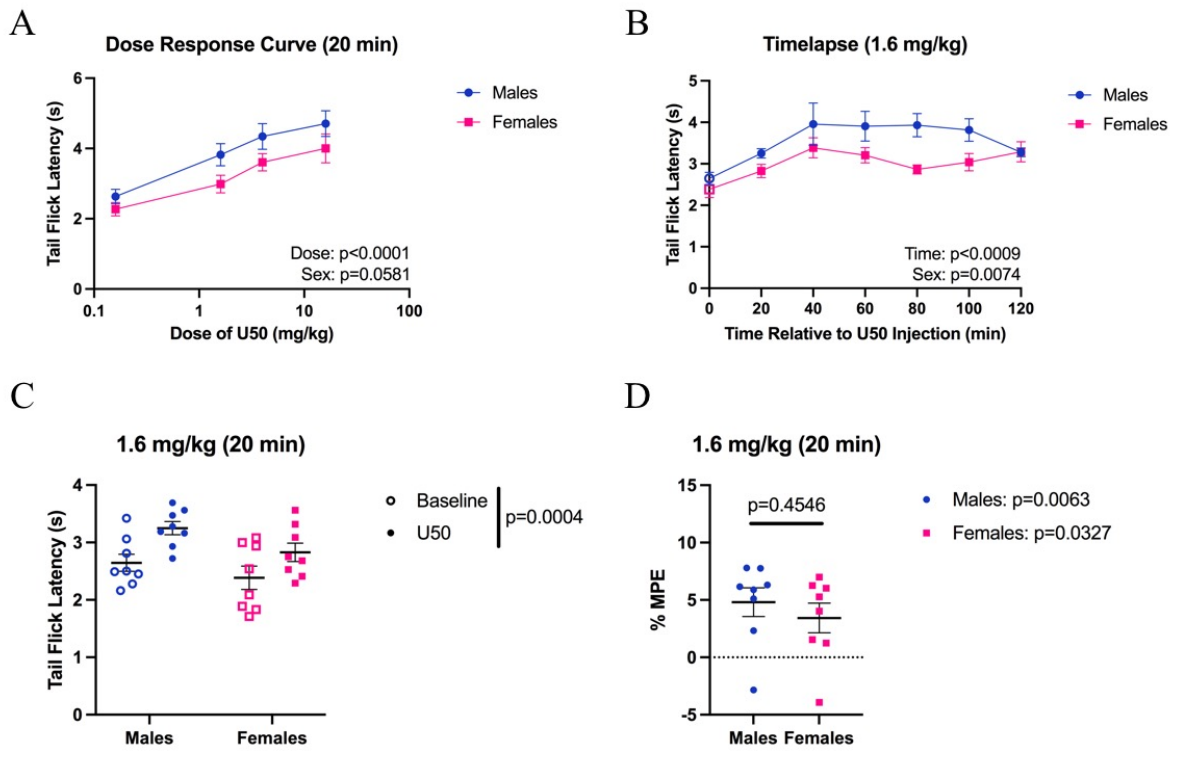

Figure 3: Systemic KOR agonism is analgesic in naïve mice. (A-D) Thermal tail withdrawal threshold testing in naïve males and females. (A) Dose response curve at 20 minutes post-injection. Increasing doses of U50 increase tail flick latency in males and females. (B) Analgesic time-course with 1.6 mg/kg U50, i.p.. Tail flick latency significantly increases over time. (C) Baseline tail flick latencies versus 20 minutes after 1.6 mg/kg of U50. U50 significantly increases tail flick latencies from baseline. No significant effect of sex was observed. (Males-Baseline: 2.646 +/- 0.149; Females-Baseline: 2.385 +/- 0.202; Males-U50: 3.250 +/- 0.114; Females-U50: 2.829 +/- 0.160). (D) The same data presented as a percentage of maximum possible effect (%MPE). U50 significantly increased the %MPE compared to baseline in both males (4.813 +/- 1.251) and females (3.430 +/- 1.292). Data presented as means +/- SEM. For full statistical details, see Appendix B.

3.2.2 Systemic KOR agonism is analgesic in EAE, but intrathecal KOR analgesia is impaired

After establishing paw withdrawal thresholds at disease onset in the EAE model, responses to U50 were tested between CFA and EAE groups (Figure 4a). As expected, CFA animals had significantly increased paw withdrawal thresholds after both low (1.6 mg/kg, i.p.; Figure 4b) and high (30 mg/kg, i.p.; Figure 4c) doses of systemic U50 compared to saline injected animals (1.6

mg/kg: Tukey's multiple comparisons, q(32)=4.590, p=0.0139; 30 mg/kg: Tukey's multiple comparisons, q(22)=11.58, p<0.0001). The same effects were seen in EAE animals (Figures 4b-c; 1.6 mg/kg: Tukey's multiple comparisons, q(32)=4.689, p=0.0116; 30 mg/kg: Tukey's multiple comparisons, q(22)=7.012, p=0.0003). At both doses, disease did not alter the effectiveness of the drug (Figures 4b-c; 1.6 mg/kg: two-way ANOVA, interaction, F(1,32)=0.03209, p=0.8590; 30 mg/kg: two-way ANOVA, interaction, F(1,22)=1.410, p=0.2478). Sex also did not alter these results (data not shown; 1.6 mg/kg: three-way ANOVA, sex, F(1,28)=0.9616, p=0.3352; 30 mg/kg: three-way ANOVA, sex, F(1,18)=0.6251, p=0.4395). To test whether this analgesia was occurring in the spinal cord, a separate cohort of EAE and CFA animals underwent paw withdrawal threshold testing after intrathecal U50 injection (10 ug U50 in 10 uL saline). The CFA animals showed a significant increase in paw withdrawal thresholds after intrathecal U50 (Figure 4d; one sample t test, theoretical mean: 0, t(9)=3.348, p=0.0085). However, the EAE animals did not show any change in paw withdrawal thresholds after intrathecal U50 (Figure 4d; one sample t test, theoretical mean: 0, t(8)=1.688, p=0.1300). Sex did not play a role in these results (data not shown; two-way ANOVA, sex, F(1,15)=0.01070, p=0.9190). This indicate that EAE significantly reduces spinal U50 analgesia, without reducing systemic U50 analgesia.

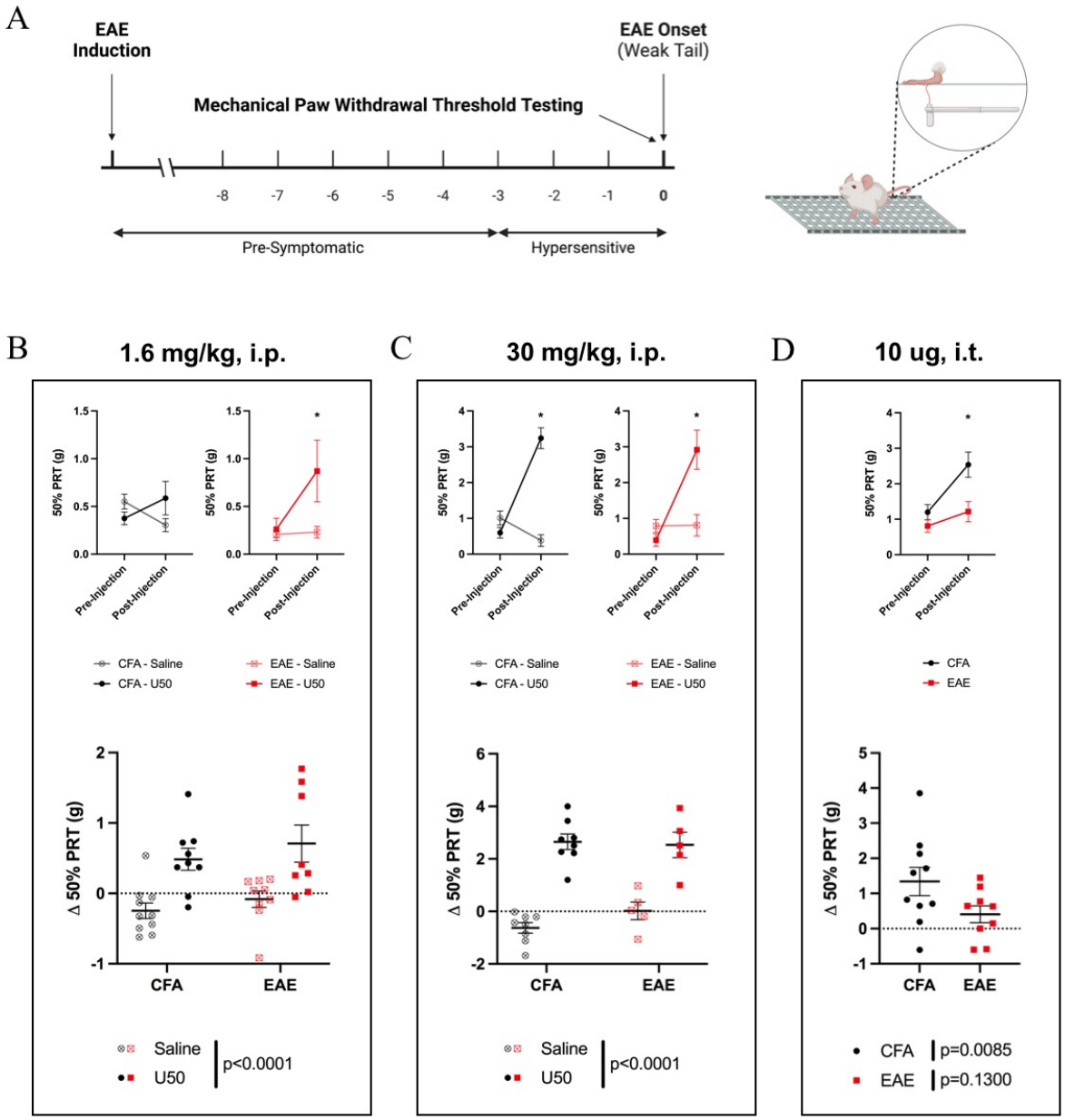

Figure 4: Systemic KOR agonism is analgesic in EAE, but intrathecal KOR analgesia is impaired. (A) Upper left: mechanical paw withdrawal thresholds at onset of CFA mice before and after intraperitoneal U50 (1.6 mg/kg) or saline. Upper right: mechanical paw withdrawal thresholds at onset of CFA mice before and after intraperitoneal U50 (1.6 mg/kg) or saline. Lower: data plotted and analyzed as change from pre-injection. Systemic low dose U50 significantly increases 50% PRT in both CFA (0.484 +/- 0.156) and EAE (0.708 +/- 0.263). Saline injections did not alter 50% PRT (CFA: -0.247 +/- 0.110; EAE: -0.082 +/- 0.116). (B) Upper: 50% PRTs of CFA (left) and EAE (right) mice before and after i.p. U50 (30 mg/kg) or saline. Lower: data plotted and analyzed as change from pre-injection. Systemic high dose U50 significantly increases 50% PRT in both CFA (2.644 +/- 0.297) and EAE (2.527 +/- 0.486).

Saline injections did not alter 50% PRT (CFA: -0.630 +/- 0.197; EAE: 0.018 +/- 0.333). (C) Upper: mechanical paw withdrawal thresholds at onset of CFA and EAE mice before and after intrathecal U50 (10 ug). Lower: data plotted and analyzed as change from pre-injection. Intrathecal U50 significantly increases 50% PRT in CFA mice (1.340 +/- 0.400) but not in EAE mice (0.4057 +/- 0.240). Significance indicated by * ($p<0.05$). Data presented as means +/- SEM. For full statistical details, see Appendix B.

3.2.3 Systemic KOR agonism is not aversive in EAE

To assess the aversive properties of KOR agonism, EAE and CFA animals underwent a conditioned place aversion (CPA) assay to a moderate dose of U50 (10 mg/kg, i.p.; Figure 5a). Individual changes in time spent in the U50-paired chamber between baseline and test day are plotted in Figures 5b-c. Data was analyzed as time spent in the U50-paired chamber on the final day of the assay (Figure 5d). The dashed line indicates spending exactly 50% of the time in each chamber (i.e., no preference or aversion). As expected, CFA animals spent significantly less than half of the time in the U50-paired chamber, indicating aversion to the drug (Figure 5d; one sample t test, theoretical mean: 900s, $t(8)=4.472$, $p=0.0021$). However, the EAE animals did not show any aversion (or preference) for the U50-paired chamber (Figure 5d; one sample t test, theoretical mean: 900s, $t(9)=0.06723$, $p=0.9479$). Directly comparing CFA and EAE results also yielded a significant difference (unpaired t test, $t(17)=2.214$, $p=0.0408$). This difference was not accounted for by any locomotor differences due to EAE, as CFA and EAE mice traveled the same amount of distance within the chambers (Figure 5e; unpaired t test, $t(17)=0.5352$, $p=0.5994$). Interestingly, there was a significant sex difference in these data (Supplementary Figure 1; two-way ANOVA, sex, $F(1,15)=11.29$, $p=0.0043$). While female CFA and EAE mice showed the same pattern as the pooled means (CFA: one sample t test, theoretical mean: 900s, $t(4)=4.82$, $p=0.0085$; EAE: one sample t test, theoretical mean: 900s, $t(4)=0.9864$, $p=0.3798$), male CFA mice showed no aversion to the U50-paired chamber (one sample t test, theoretical mean: 900s, $t(4)=0.1550$, $p=0.8843$) and

male EAE mice showed a significant preference for the U50-paired chamber (one sample t test, theoretical mean: 900s, t(3)=4.443, p=0.0212). Collectively, these data suggest that EAE impairs U50 aversion.

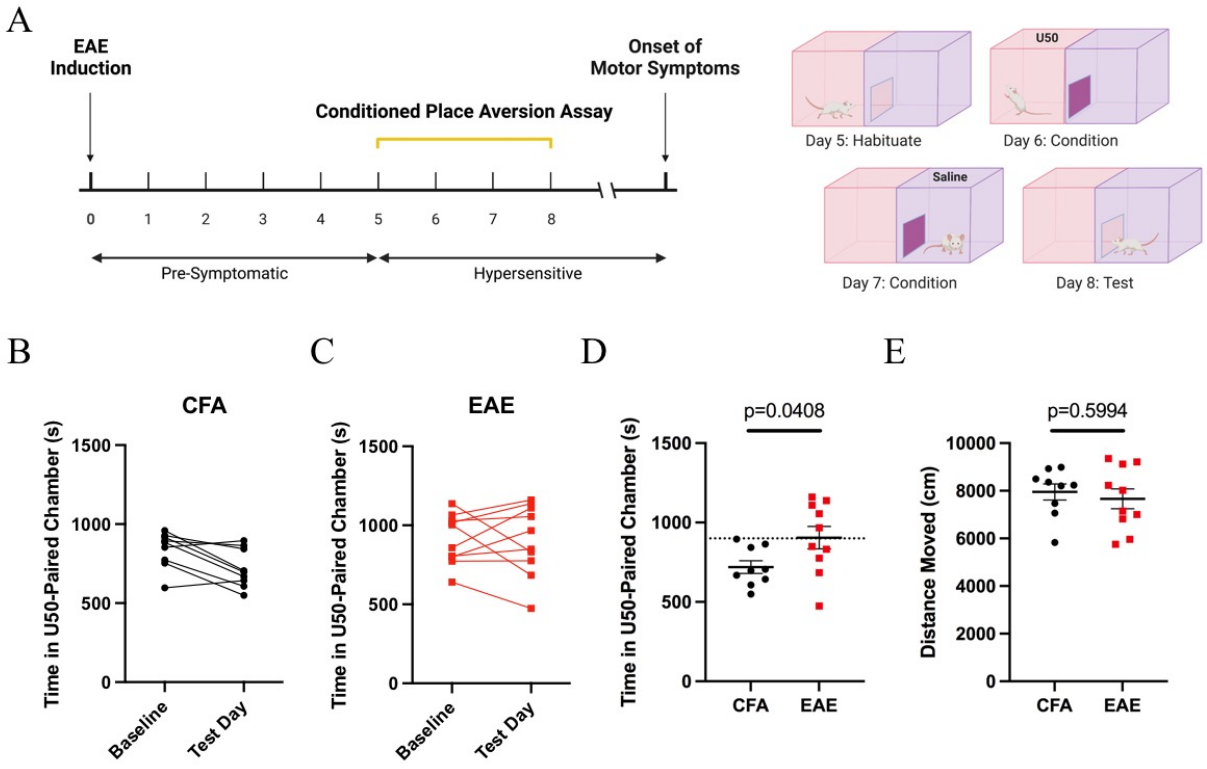

Figure 5: Systemic KOR agonism is not aversive in EAE. (A) Timeline and schematic diagram for conditioned place aversion assay. (B) Changes in time spent in U50-paired chamber of individual CFA mice between baseline (Day 5) and test day (Day 8). (C) Changes in time spent in U50-paired chamber of individual EAE mice between baseline and test day. (D) Time spent in U50-paired chamber on test day. Dotted line indicates half of the time available to explore (i.e., no preference or aversion). CFA mice spent significantly less than half their time in the U50-paired chamber (719.3 +/- 40.41), which was significantly less than EAE mice, who spent equal time in both chambers (904.7 +/- 70.51). (C) Distance moved on test day. CFA (7953 +/- 336.9) and EAE (7661 +/- 418.8) mice moved around to the same extent. Data presented as means +/- SEM. For full statistical details, see Appendix B.

3.3 Changes in the Kappa Opioid System in EAE

3.3.1 KOR mRNA and protein levels are downregulated in the spinal cord in EAE

Lumbar spinal cord tissue from CFA and EAE mice were analyzed via western blot for KOR protein expression. At disease onset, there was no difference in KOR protein expression in EAE compared to CFA mice (Figure 6a; unpaired t test, $t(32)=0.07477$, $p=0.9409$). There was no significant effect of sex on KOR protein expression (data not shown; two-way ANOVA, sex, $F(1,30)=2.311$, $p=0.1389$). When the western blot was repeated on lumbar spinal cords collected at day 7 post-onset ("chronic"), there was a significant decrease in the expression of KOR protein in EAE compared to CFA mice (Figure 6b; Mann-Whitney, $U=45$, $p=0.0255$). There was no significant effect of sex or daily U50 treatment on KOR protein expression (data not shown; three-way ANOVA, sex, $F(1,19)=0.1160$, $p=0.7371$; three-way ANOVA, treatment, $F(1,19)=0.02737$, $p=0.8703$). Lumbar spinal cord sections from CFA and EAE mice at day 7 post-onset were analyzed via fluorescent *in situ* hybridization (FISH) for *Oprk1* and *Pdyn* mRNA expression (Figure 6c; representative images). EAE mice showed a significant decrease in *Oprk1* mRNA in the dorsal horn as compared to CFA mice (Figure 6d; unpaired t test, $t(26)=3.513$, $p=0.0016$). *Pdyn* mRNA expression was unchanged in the dorsal horn of EAE mice compared to CFA mice (Figure 6e; Mann-Whitney, $U=65$, $p=0.1393$). There was no significant effect of sex or daily U50 treatment in *Oprk1* (data not shown; three-way ANOVA, sex, $F(1,19)=0.3276$, $p=0.5738$; three-way ANOVA, treatment, $F(1,19)=0.1823$, $p=0.6742$) or *Pdyn* mRNA expression (data not shown; three-way ANOVA, sex, $F(1,19)=0.6824$, $p=0.4190$; three-way ANOVA, treatment, $F(1,19)=0.1845$, $p=0.6723$). When this data was broken down into SDH versus DDH, EAE significantly downregulated *Oprk1* mRNA levels in both the SDH (Supplementary Figure 2; Welch's t test, $t(25.97)=4.957$, $p<0.0001$) and the DDH (Supplementary Figure 2; unpaired t test,

t(32)=3.100, p=0.0040). In the SDH, *Pdyn* mRNA was still unchanged by EAE (Supplementary Figure 2; Mann-Whitney, U=106, p=0.1310), however, a significant downregulation of *Pdyn* mRNA by EAE emerged in the DDH (Supplementary Figure 2; Mann-Whitney, U=83, p=0.0219). Together, these data show that EAE decreases KOR mRNA and protein in the spinal cord.

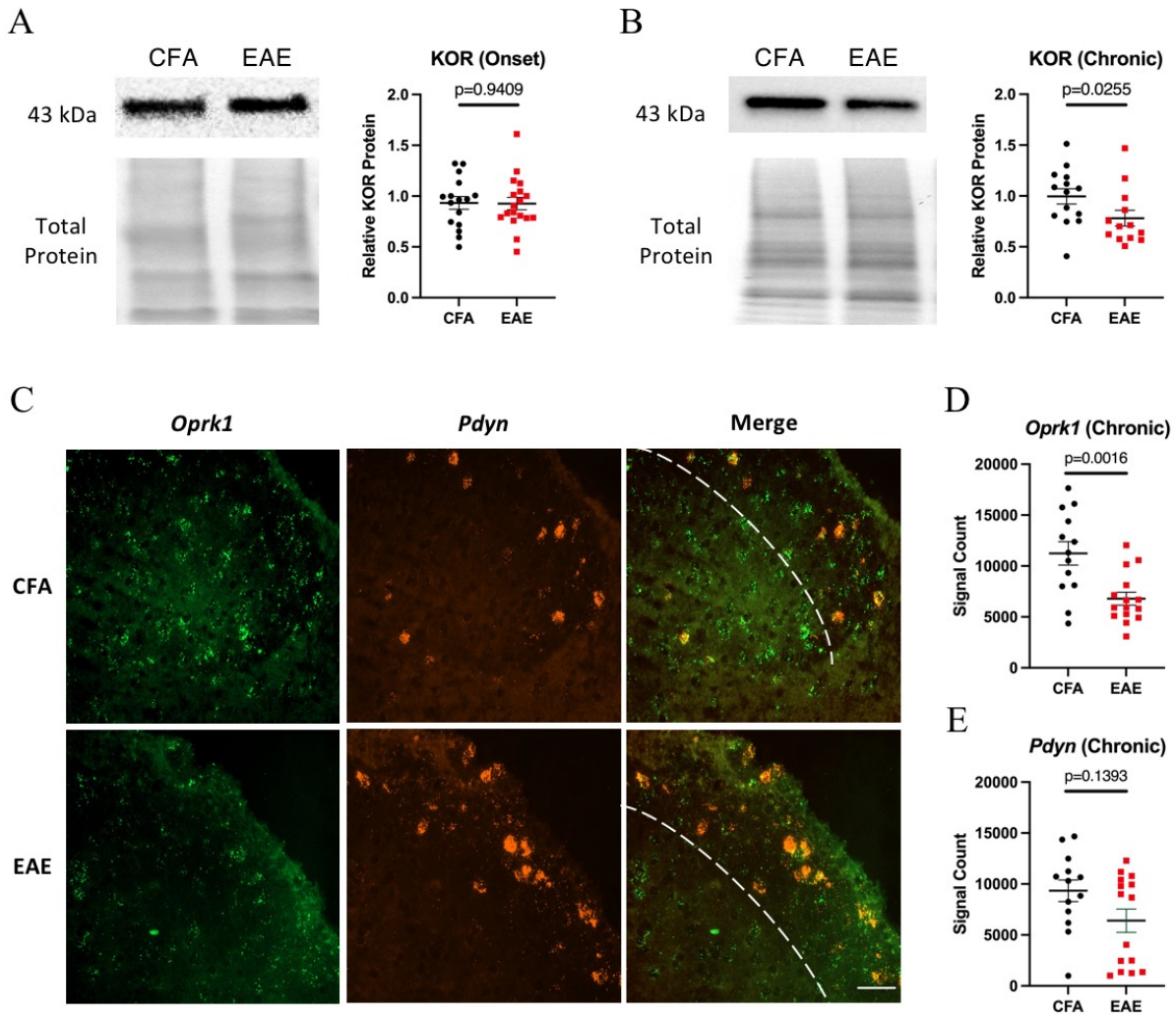

Figure 6: KOR mRNA and protein levels are downregulated in the spinal cord in EAE. (A-B) Western blot analysis of KOR protein levels in the lumbar spinal cord with representative bands and total protein control. (A) At onset, there was no difference in spinal KOR protein levels between CFA (0.9332 +/- 0.619) and EAE (0.9266 +/- 0.0614). (B) At 7 days post-onset, EAE mice (0.7812 +/- 0.0772) showed significantly lower spinal KOR protein levels compared to CFA mice (0.9963 +/- 0.0747). (C-E) FISH analysis of *Oprk1* and *Pdyn* mRNA in the dorsal horn of the lumbar spinal cord at 7 days post-onset. (C) Representative images at 40x. Dashed lines on merged images

indicate approximate division between SDH (upper right) and DDH (lower left). (D) EAE (6790 +/- 638.3) significantly decreases spinal *Oprk1* signal count compared to CFA (11241 +/- 1147). (E) EAE (6403 +/- 1138) does not change spinal *Pdyn* signal count compared to CFA (9336 +/- 1053). Data presented as mean +/- SEM. Scale bar = 50 um. For full statistical details, see Appendix B.

3.3.2 KOR protein levels are upregulated in the nucleus accumbens in EAE

Western blot analysis of NAc tissue at onset showed a significant increase in KOR protein levels in EAE compared to CFA (Figure 7b; Mann-Whitney, U=51, p=0.0003). Although these animals did not undergo daily U50 treatment, half of them received 30 mg/kg of U50 (i.p.) approximately one hour prior to tissue extraction. Interestingly, only the EAE animals that received U50 showed this increase in KOR protein in the NAc (Supplementary Figure 3; two-way ANOVA, interaction, $F(1,32)=35.15$, $p<0.0001$). NAc tissue from EAE and CFA mice at onset was assessed for *Oprk1* and *Pdyn* mRNA expression in the NAcC (Figure 7c) and NAcSh (Figure 7f). There was no significant alterations in *Oprk1* or *Pdyn* mRNA expression in the NAcC (Figures 7d-e; *Oprk1*: unpaired t test, $t(16)=0.1268$, $p=0.9007$; *Pdyn*: unpaired t test, $t(16)=0.08379$, $p=0.9343$). Similar results were seen in the NAcSh (Figures 7g-h; *Oprk1*: unpaired t test, $t(16)=0.7454$, $p=0.4668$; *Pdyn*: unpaired t test, $t(16)=1.372$, $p=0.1891$). There was no significant effect of sex on the expression of *Oprk1* or *Pdyn* mRNA in the NAcC (data not shown; *Oprk1*: two-way ANOVA, sex, $F(1,14)=2.433$, $p=0.1411$; *Pdyn*: two-way ANOVA, sex, $F(1,14)=0.2176$, $p=0.6481$) or in the NAcSh (data not shown; *Oprk1*: two-way ANOVA, sex, $F(1,14)=0.9216$, $p=0.3533$; *Pdyn*: two-way ANOVA, sex, $F(1,14)=0.5759$, $p=0.4605$). Overall, these data reveal that KOR protein is significantly increased in EAE. These changes are not reflected in mRNA levels.

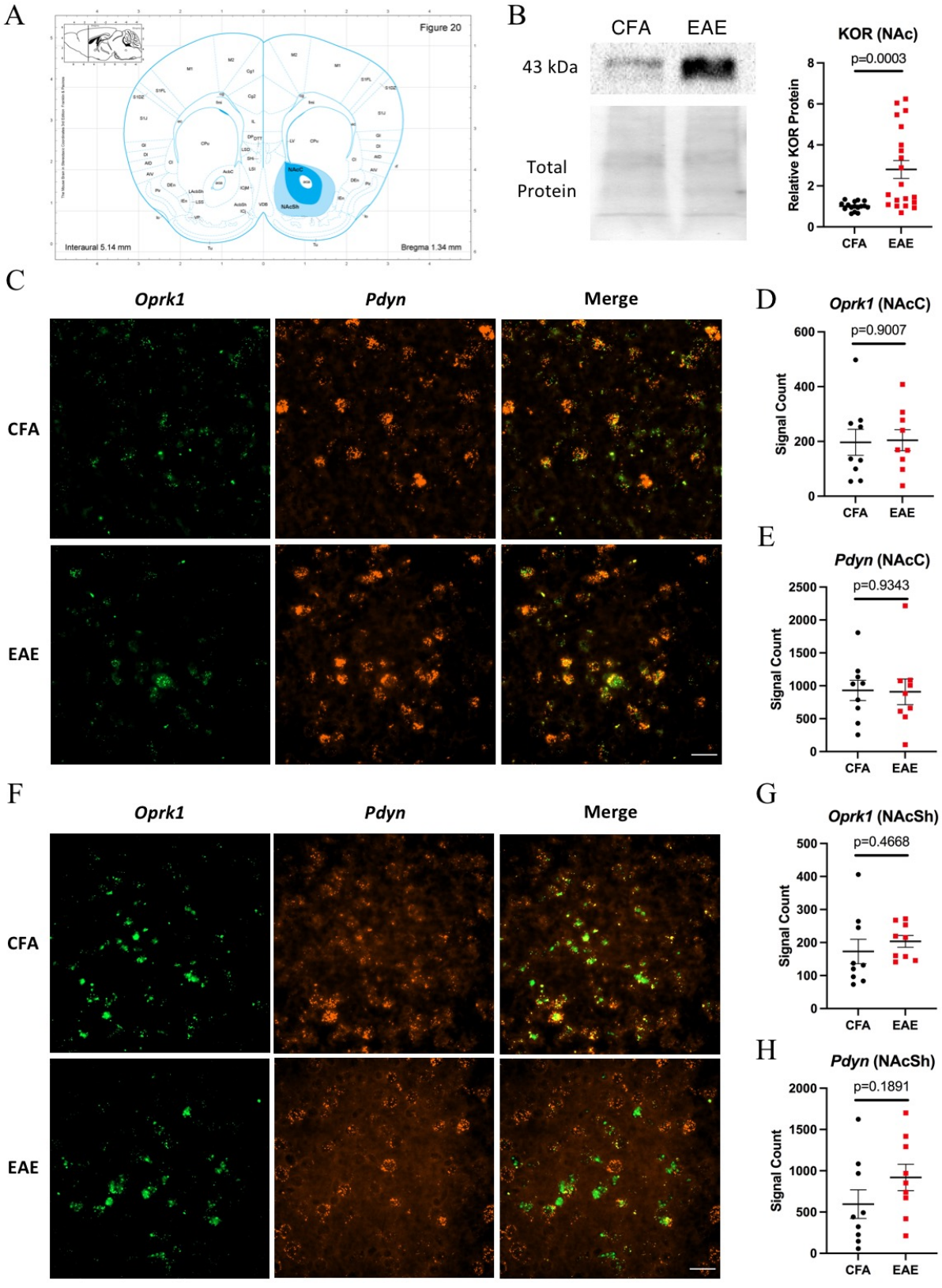
A
Figure 20
Interaural 5.14 mm
Bregma 1.34 mm
B
CFA
EAE
43 kDa
Total Protein
KOR (NAc)
p=0.0003
Relative KOR Protein
C
Oprk1
Pdyn
Merge
CFA
EAE
D
Oprk1 (NAcC)
p=0.9007
Signal Count
E
Pdyn (NAcC)
p=0.9343
F
G
Oprk1 (NAcSh)
p=0.4668
H
Pdyn (NAcSh)
p=0.1891

Figure 7: KOR protein levels are upregulated in the nucleus accumbens in EAE. (A) Schematic diagram showing the demarcation between NAcC and NAcSh (modified from Allen Mouse Brain Atlas). (B) Western blot analysis of KOR protein levels in the NAc at onset with representative bands and total protein control. EAE mice (2.801 +/- 0.437) showed significantly higher NAc KOR protein levels compared to CFA mice (1.011 +/- 0.544). (C-E). FISH analysis of *Oprk1* and *Pdyn* mRNA in the NAcC at onset. (E) Representative images of the NAcC at 63x. EAE does not change NAcC *Oprk1* signal count (E; 204.4 +/- 38.19) nor *Pdyn* signal count (F; 908.7 +/- 194.3) compared to CFA (*Oprk1*: 196.7 +/-47.52; *Pdyn*: 929.5 +/- 154.5). (F-H). FISH analysis of *Oprk1* and *Pdyn* mRNA in the NAcSh at onset. (G) Representative images of the NAcSh at 63x. EAE does not change NAcSh *Oprk1* signal count (H; 203.5 +/- 17.87) nor *Pdyn* signal count (I; 919.6 +/- 160.2) compared to CFA (*Oprk1*: 173.0 +/- 36.82; *Pdyn*: 595.5 +/- 173.7). Data presented as mean +/- SEM. Scale bar = 25 um. For full statistical details, see Appendix B.

3.3.3 KOR mRNA and protein levels are unchanged in the amygdala in EAE

At onset, amygdala KOR protein expression at onset was not significantly different between CFA and EAE mice (Figure 8b; unpaired t test, t(35)=0.8122, p=0.4222). These results were unaffected by sex or acute high-dose U50 treatment (data not shown; three-way ANOVA, sex, F(1,29)=0.2222, p=0.6409; three-way ANOVA, treatment, F(1,29)=1.055, p=0.3129). FISH analysis of *Oprk1* and *Pdyn* mRNA expression at onset was analyzed in the CeA (Figure 8c) and the BLA (Figure 8f). *Oprk1* and *Pdyn* mRNA expression were unchanged in the CeA of EAE compared to CFA animals (Figures 8d-e; *Oprk1*: unpaired t test, t(18)=0.1286, p=0.8991; *Pdyn*: unpaired t test, t(18)=0.6517, p=0.5228). However, sex and disease did interact to significantly alter *Oprk1* (Supplementary Figure 4; two-way ANOVA, sex x disease interaction, F(1,16)=8.647, p=0.0096) and *Pdyn* (Supplementary Figure 4; two-way ANOVA, sex x disease interaction, F(1,16)=4.804, p=0.0435) mRNA levels. In the BLA, *Oprk1* and *Pdyn* mRNA expression was not significantly different between CFA and EAE animals (Figures 8g-h; *Oprk1*: Mann-Whitney, U=43.50, p=0.9215; *Pdyn*: unpaired t test, t(18)=1.059, p=0.3038). Unlike the CeA, there was no significant effect of sex on *Oprk1* mRNA levels in the BLA of CFA (data not shown; multiple Mann-Whitney, U=8, p=0.730159) or EAE (data not shown; multiple Mann-Whitney, U=11,

p=0.841270) animals. This was also the case for *Pdyn* mRNA expression in the BLA (data not shown; two-way ANOVA, sex, F(1,16)=0.4509, p=0.5115). In summary, EAE does not affect amygdalar KOR mRNA or protein expression.

3.3.4 Prodynorphin mRNA levels are downregulated in the claustrum in EAE

Western blot analysis did not show any significant changes to KOR protein expression in the claustrum of EAE compared to CFA mice (Figure 9b; Mann-Whitney, U=28, p=0.5362). Sex was not a significant factor in CFA or EAE mice (data not shown; multiple Mann-Whitney, CFA, U=0, p=0.095238; multiple Mann-Whitney, EAE, U=10, p=0.690476). Claustrum tissue from EAE and CFA animals at onset were analyzed for *Oprk1* and *Pdyn* mRNA expression (Figure 9c). While *Oprk1* mRNA expression did not show any changes in EAE mice compared to CFA (Figure 9d; unpaired t test, t(17)=0.7046, p=0.4906), *Pdyn* mRNA was significantly downregulated in EAE compared to CFA mice (Figure 9e; Welch's t test, t(12.77)=2.776, p=0.0160). Sex was not a significant factor when it came to *Oprk1* (data not shown; two-way ANOVA, sex, F(1,15)=0.3881, p=0.5426) or *Pdyn* mRNA expression (data not shown; two-way ANOVA, sex, F(1,15)=0.01254, p=0.9123). Similar to the amygdala, EAE did not alter KOR mRNA or protein levels in the claustrum.

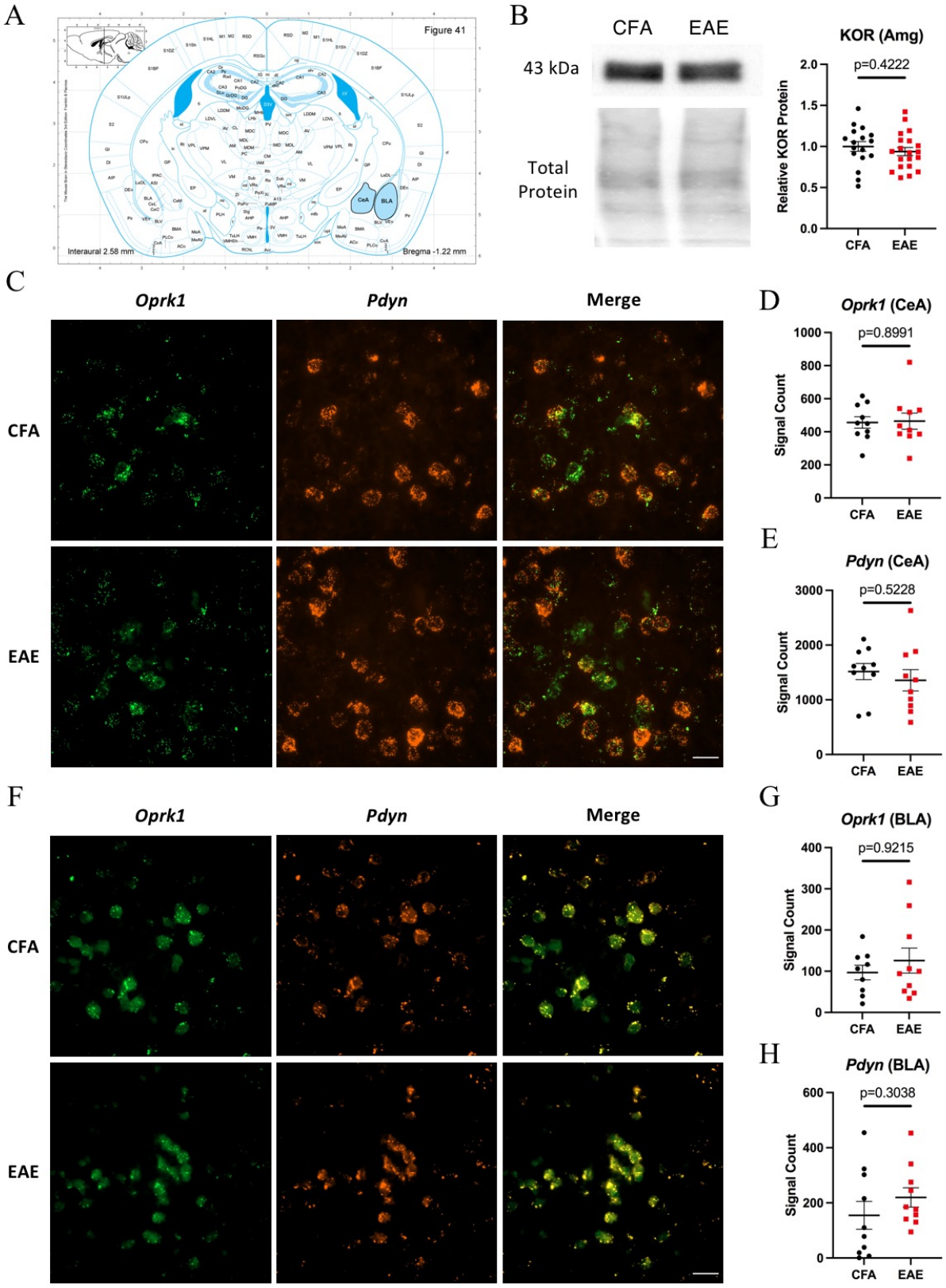
A
Figure 41
Interaural 2.58 mm
Bregma -1.22 mm
CeA
BLA
B
CFA
EAE
43 kDa
Total Protein
KOR (Amg)
p=0.4222
Relative KOR Protein
2.0
1.5
1.0
0.5
0.0
CFA
EAE
C
Oprk1
Pdyn
Merge
CFA
EAE
D
Oprk1 (CeA)
p=0.8991
Signal Count
1000
800
600
400
200
0
CFA
EAE
E
Pdyn (CeA)
p=0.5228
Signal Count
3000
2000
1000
0
CFA
EAE
F
Oprk1
Pdyn
Merge
CFA
EAE
G
Oprk1 (BLA)
p=0.9215
Signal Count
400
300
200
100
0
CFA
EAE
H
Pdyn (BLA)
p=0.3038
Signal Count
600
400
200
0
CFA
EAE

Figure 8: KOR mRNA and protein levels are unchanged in the amygdala in EAE. (A) Schematic diagram showing the demarcation between CeA and BLA (modified from Allen Mouse Brain Atlas). (B) Western blot analysis of KOR protein levels in the amygdala at onset with representative bands and total protein control. EAE (0.9370 +/- 0.050) does not change amygdalar KOR protein levels compared to CFA (0.9995 +/- 0.059). (C-E). FISH analysis of *Oprk1* and *Pdyn* mRNA in the CeA at onset. (C) Representative images of the CeA at 63x. EAE does not change CeA *Oprk1* signal count (D; 464.3 +/- 48.76) nor *Pdyn* signal count (E; 1357 +/- 194.8) compared to CFA (*Oprk1*: 456.6 +/- 34.78; *Pdyn*: 1516 +/- 147.5). (F-H). FISH analysis of *Oprk1* and *Pdyn* mRNA in the BLA at onset. (F) Representative images of the BLA at 63x. EAE does not change BLA *Oprk1* signal count (G; 125.7 +/- 30.39) nor *Pdyn* signal count (H; 219.8 +/- 34.95) compared to CFA (*Oprk1*: 96.84 +/- 17.56; *Pdyn*: 154.9 +/- 50.43). Data presented as mean +/- SEM. Scale bar = 25 um. For full statistical details, see Appendix B.

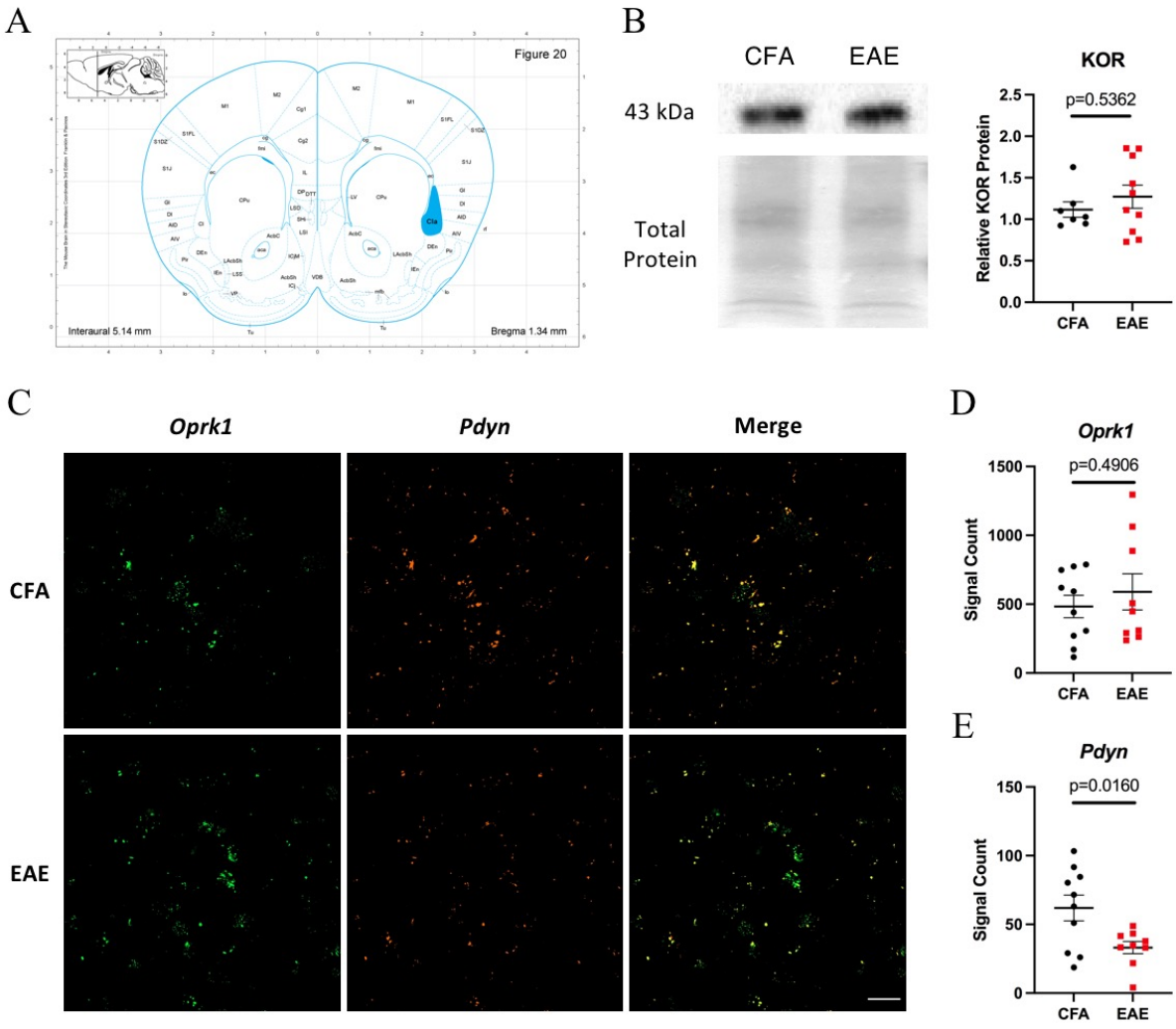

Figure 9: Prodynorphin mRNA levels are downregulated in the claustrum in EAE. (A) Schematic diagram locating the claustrum (modified from Allen Mouse Brain Atlas). (B) Western blot analysis of KOR protein levels in the claustrum at onset with representative bands and total protein control. EAE (1.272 +/- 0.140) does not change claustrum KOR protein levels compared to CFA (1.117 +/- 0.092). (C-E). FISH analysis of *Oprk1* and *Pdyn* mRNA in the claustrum at onset. (C) Representative images of the claustrum at 63x. EAE does not change claustrum *Oprk1* signal count (E; 589.0 +/- 131.1) nor *Pdyn* signal count (F; 33.11 +/- 4.44) compared to CFA (*Oprk1*: 482.9 +/- 80.9; *Pdyn*: 61.89 +/- 9.37). Data presented as mean +/- SEM. Scale bar = 25 um. For full statistical details, see Appendix B.

CHAPTER 4: Discussion

This thesis was designed to investigate the kappa opioid system in a mouse model of MS and its involvement in the sensory, affective, and cognitive aspects of pain. Firstly, I found that daily administration of selective KOR agonist U50 (1.6 mg/kg, i.p.) did not significantly modify EAE disease progression when treatment began at symptom onset. The analgesic potential of U50 (as seen in naïve mice), was conserved in EAE mice at low and high doses when administered intraperitoneally. However, intrathecal injection did not provide significant analgesia in EAE mice. The aversive nature of U50 was also altered in EAE, with only the CFA mice displaying signs of aversion. Next, I found that KOR mRNA and protein levels were decreased in the lumbar spinal cord of EAE mice. Conversely, KOR protein expression, but not mRNA expression, was increased in the NAc of EAE mice. Neither KOR mRNA nor protein were significantly altered by EAE in the amygdala and claustrum. Pre-prodynorphin mRNA was significantly downregulated in the claustrum, and unchanged in all other regions analyzed. Overall, these results indicate that EAE leads to reduced KOR expression in the spinal cord, and this correlates with impaired spinal analgesia of KOR agonists. Interestingly, brain KOR expression exhibited a different pattern of expression in EAE, either being unchanged (claustrum, amygdala) or significantly increased (NAc). The behavioural ramifications of these brain level changes remain unclear, and are an area of future investigation.

The first result of particular interest was in relation to the effect of repeated KOR agonism on EAE disease course. While the previous literature demonstrates that daily injection of U50 (1.6 mg/kg, i.p.) significantly reduces EAE severity when treatment begins 3 days post-induction (i.e., in the pre-symptomatic phase) (Du et al., 2016), my results show that delaying treatment until symptom onset prevents this therapeutic effect. A newly published paper followed the same

protocol as I did, and found that daily U50 treatment (1.6 mg/kg, i.p.) beginning at onset did begin to show improved disease progression from 1.5-3 weeks post-onset (Denny et al., 2021). However, like my results, there was no difference in EAE scores in the first week of treatment. This is likely because KOR agonism in EAE promotes remyelination (Du et al., 2016; Mei et al., 2016; Wang & Mei, 2019), which typically begins in the second week of EAE, whereas the first week is the demyelination phase, as evidenced by the progressive motor impairment. Beginning treatment at onset was too late to reduce the severity of the demyelination phase, like Du et al. (2016) were able to accomplish with prophylactic treatment. Overall, my results provide evidence that the there is a specific therapeutic window for KOR agonist therapy in different phases of EAE progression. These results also allowed me to investigate the analgesic and aversive effectiveness of KOR agonism without the potential confound of an altered disease course.

My behavioural pain assays showed that the response to KOR agonism is altered in EAE. Although systemic U50 administration was effectively analgesic in CFA and EAE mice, intrathecal U50 administration was only effective in CFA mice. This suggests that the analgesia produced by systemic U50 is not attributable to the spinal cord in EAE mice. One possible explanation is that the analgesia is caused by activation of KORs in the periphery. The KOR is present on the peripheral terminals and cell bodies of the nociceptors that synapse in the spinal cord, and activation of these receptors via peripherally-restricted KOR agonists does produce antinociception (Albert-Vartanian et al., 2016; Obara et al., 2009; Rivière, 2004; Snyder et al., 2018). However, we do not yet know how the analgesic potential of peripherally restricted KOR agonists is affected in EAE. Another possible explanation is the engagement of supraspinal analgesic pathways. This idea is supported by a study that showed that that systemic KOR agonism

produced analgesia that was unaffected by peripherally-restricted KOR antagonism and only partially blocked by intrathecal KOR antagonism (Millan et al., 1989). One such supraspinal mechanism is stress-induced analgesia, since blocking stress with diazepam reduces the analgesic effect of U50 (Taylor et al., 2015). While more research is required, these results could suggest that EAE recruits stress induced analgesic pathways that compensate for the loss of spinal antinociception. Whether peripherally or supraspinally driven, these data rule out the spinal cord as the primary site of antinociceptive action of systemic U50 in EAE.

This lack of intrathecal U50 antinociception lines up with the reduction in KOR mRNA and protein expression that I found in the lumbar spinal cord. These results match the downregulation of KOR mRNA seen in the spinal cords of mice affected with Theiler's murine encephalomyelitis virus – an alternative model of MS (Lynch et al., 2008). Conversely, the lack of a significant change in pre-prodynorphin mRNA in the spinal cord does not match the chronic pain literature. As outlined in the introduction of this thesis, various models of chronic pain all result in increased spinal dynorphin levels (Faden et al., 1985; Millan et al., 1986, 1988; Wagner et al., 1993). However, this study showed that EAE-related pain does not increase dynorphin levels, and instead may even have decreased spinal *Pdyn* mRNA in a subset of EAE mice (Figure 6g). The bimodal distribution of *Pdyn* expression in EAE mice could not be correlated or attributed to any discernible variable, including sex, U50 treatment, paw withdrawal thresholds, or EAE scores (data not shown). Despite the spinal cord data not lining up with the chronic pain literature, it may make sense with the myelination data outlined above; increased exogenous KOR agonism could be having a beneficial effect in EAE because endogenous KOR agonism is decreased by the downregulation of KOR protein. If this is the case, it would indicate a more causal role of KOR

dysregulation in EAE progression rather than a symptomatic role, but the nature of this relationship is merely speculation at this point and would need to be directly tested. In the same manner, it could be the downregulation of spinal KOR that is driving the state of sustained pain, since its inhibitory effect on nociception is lost. Again, this would require further experimentation to elucidate the directionality of this association.

Another aspect of my study that provides an avenue for further research is the CPA data. The fact that the EAE mice do not display any aversion toward U50 has many possible explanations. The idea that EAE could impair locomotion and confound the time spent in each chamber was controlled for by performing the assay before the onset of motor symptoms and confirmed to not be a factor by the data showing no difference in distance moved on the assay's test day. The possibility of the lack of aversion in EAE being a result of a deficit in associative learning is rejected by the fact that EAE mice display aversion to a sub-therapeutic dose of morphine in the same CPA assay (Dworsky-Fried et al., 2021). As for explanations that cannot so easily be refuted, one possibility is that there has been some sort of expression level or functional change in KOR circuitry that has made KOR agonism less aversive in EAE mice. However, a more likely explanation is that the benefit of KOR-mediated analgesia has balanced out the cost of KOR-mediated aversion. This explanation lines up with data showing that in a CPA assay, mice with peripheral nerve injury found increasing doses of U50 to be less aversive (and more analgesic), while naïve and sham mice found increasing doses of U50 to be more aversive (Liu et al., 2019). Together, these data reflect the complex decision-making process performed by mice while navigating the CPA assay. It suggests that mice in pain will not avoid the U50-paired chamber because they experience some positive analgesic effect following U50 treatment. Pain-naïve mice

will experience no analgesic benefit to offset the aversive aspect of U50, leading to avoidance of the U50-paired chamber. In terms of clinical translatability, a painkiller that provides no benefit to an individual that is not in pain is a promising development within the current addiction crisis. However, more pointed experiments are required in order to explore that implication further.

The next results that deserve consideration are the KOR expression data from the brain regions I analyzed. There was no significant difference in KOR mRNA or protein expression in the amygdala or claustrum, but there was a robust increase in KOR protein levels in the NAc. This increase compliments the data from inflammatory and neuropathic pain models that show an increase in KOR mRNA expression in the NAc (Liu et al., 2019; Massaly et al., 2019), although I did not see changes at the mRNA level. However, this increase in combination with the lack of U50 aversion does not line up with a study that showed that KOR activation on NAc dopaminergic terminals are responsible for KOR-mediated CPA (Chefer et al., 2013). Nevertheless, this view does not take into account the fact that the increase in NAc KOR protein in EAE may not be on the dopaminergic neurons. There is also the possibility that this upregulation of KOR protein is a compensatory mechanism for a functional decrease in KOR signaling. Future studies should parse out the reason for this increase, as well as attempt to localize the effect in regards to the NAcC versus NAcSh, and in regards to different cell populations. The reason that the increase in NAc KOR protein was only seen in EAE animals that had received an injection of U50 (30 mg/kg, i.p.) an hour before tissue collection is not immediately clear, as one would expect a high level of agonism to induce receptor internalization and degradation, rather than upregulation (Kunselman et al., 2021). However, one study found that U50 administration (5 mg/kg, i.p.) 30 minutes prior to tissue collection induced internalization of KOR, but with a concurrent upregulation of KOR

protein through western blot analysis in the locus coeruleus (Reyes et al., 2010). This brain region is known to send direct projections to the NAc, so could potentially be the source of the agonist-induced KOR upregulation, or may just point to a similar mechanism occurring in the NAc (Delfs et al., 1998). The fact that this did not occur in the CFA mice remains unclear.

It is also worth noting that mRNA expression of *Pdyn* was downregulated in the claustrum. Analyzing the kappa opioid system in the claustrum of mice with chronic pain is a novel venture that leaves no literature to compare. However, some speculation is possible. As reviewed in the Chapter 1, increases in claustrum activity have been linked to pain states, while decreases have been linked to cognitively-mediated pain reduction (Amanzio et al., 2013; Gracely et al., 2004; Palermo et al., 2015; Słoniewski et al., 1995). If we suppose that the inhibitory action of KOR agonism leads to a general decrease in claustrum activity, then a decrease in *Pdyn* expression (and consequently a decrease in KOR agonism) could lead to an increase in claustrum activity. This provides a potential mechanism for the positive association between claustrum activity and pain states. Although experiments are needed to determine whether this decrease in *Pdyn* mRNA translates to a decrease in protein levels and subsequent KOR activation, this finding opens the door to an unexplored avenue of research concerning the kappa opioid system and the claustrum in pain.

The last piece of my experiments that deserves consideration is the lack of sex differences in the collected data. MS is known to disproportionately affect women, with a patient sex ratio of 3:1 (Rankin & Bove, 2018). Chronic pain is similarly more common in women (Campbell et al., 2019). Although I saw more severe EAE scores in male mice – which agrees with the human MS

literature (Tomassini & Pozzilli, 2009) – there was no sex difference in spinal cord inflammation or mechanical allodynia at onset. A previous paper has shown the lack of sex differences in these two measures, but went on to show that the mechanism behind the pain may differ, with significantly higher astrocyte reactivity and axonal damage seen in male mice (Catuneanu et al., 2019). These sex differences are supported by differential response of pain to exercise and differential microRNA signatures in EAE (Friedman et al., 2019; Mifflin et al., 2017, 2019). My data also revealed no sex difference in the effect of U50 on paw withdrawal thresholds in CFA or EAE mice, even though KOR-mediated analgesia is often found to be less effective in females (Taylor et al., 2020), although I only tested very low and very high doses of U50. The only data in which I saw sex differences were the CPA and the *Oprk1/Pdyn* mRNA levels in the CeA (see supplemental figures). Subsequent experiments should continue to be run with an equal sex ratio in light of its potential influence.

Overall, my thesis has accomplished what it set out to examine. I was able to demonstrate changes in the function and expression of the kappa opioid system in a mouse model of MS in behaviours and CNS regions associated with the sensory, affective, and cognitive aspects of pain. Loss of KOR expression and function in the spinal cord may indicate a novel disease mechanism contributing to the disease progression and pain symptomology associated with MS. These data provide further evidence that strategies aimed at increasing KOR expression or function may be effective at treating several facets of MS. Moreover, although the kappa opioid system is altered in a model of MS, the analgesic capacity of KOR agonists is unchanged when given systemically. This, coupled with the decreased aversion to KOR agonism, suggests that KOR agonists may be a viable therapeutic option for MS-induced chronic pain and further research into this application

should be pursued. Like in the chronic pain literature, we do not know whether changes in the kappa opioid system are a cause or a consequence of this EAE-associated pain. Future investigations should attempt to answer this question in order to fully understand the relationship between the kappa opioid system and MS-induced chronic pain. This thesis has shown this to be a viable avenue of research, and has effectively begun to bridge the gap in knowledge between the kappa opioid system and the MS pain literature.

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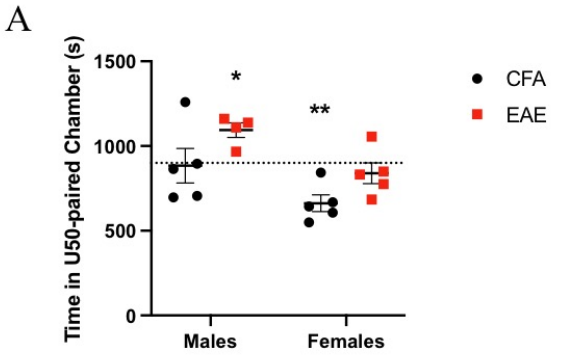
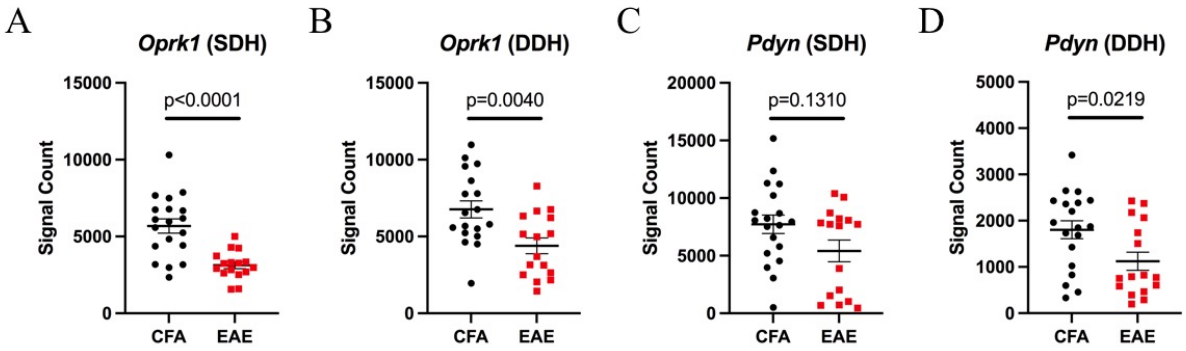
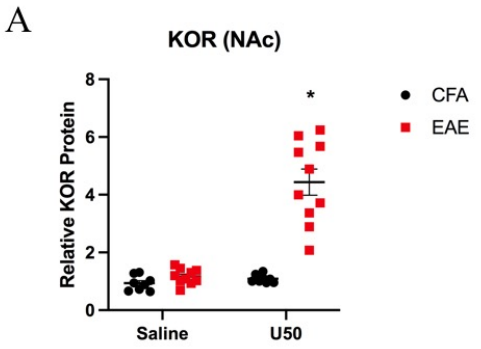
Appendix A: Supplementary Figures



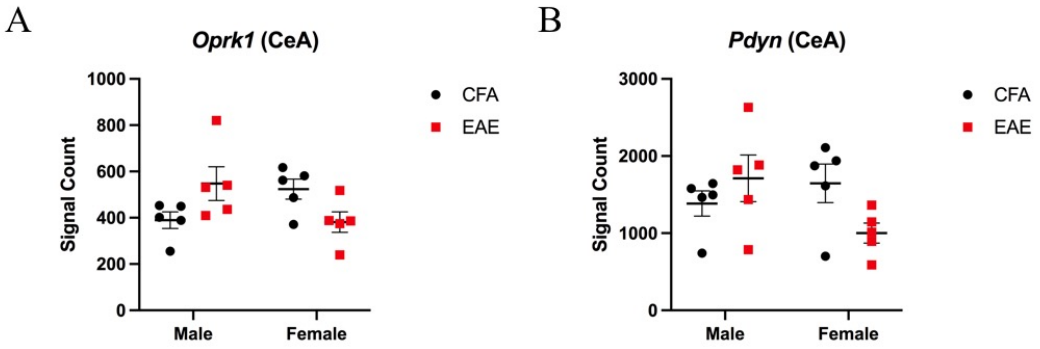
Supplementary Figure 1: **Time spent in U50-paired chamber is dependent on sex and disease.** (A) Time spent in U50-paired chamber on test day in CFA and EAE mice. Dotted line indicates half of the time available to explore (i.e., no preference or aversion). Male CFA mice spend equal time in both chambers (884.2 +/- 102.0). Male EAE mice spend significantly more than half the time in the U50-paired chamber (1094 +/- 43.6). Female CFA mice spend significantly less than half the time in the U50-paired chamber (662.3 +/- 49.3). Female EAE mice spend equal time in both chambers (839.4 +/- 61.4). *p=0.0212. **p=0.0085. Data presented as means +/- SEM. For full statistical details, see Appendix B.



Supplementary Figure 2: Prodynorphin mRNA levels are downregulated in the DDH in EAE. FISH analysis of *Oprk1* and *Pdyn* mRNA in the SDH and DDH of the spinal cord at 7 days post-onset. (A) *Oprk1* mRNA is significantly lower in the SDH of EAE mice (3135 +/- 226.9) compared to CFA mice (5679 +/- 460.2). (B) *Oprk1* mRNA is significantly lower in the DDH of EAE mice (4396 +/- 515.8) compared to CFA mice (6766 +/- 555.9). (C) *Pdyn* mRNA is not significantly changed in the SDH of EAE mice (5416 +/- 936.8) compared to CFA mice (7722 +/- 802.1). (D) *Pdyn* mRNA is significantly lower in the DDH of EAE mice (1123 +/- 197.0) compared to CFA mice (1804 +/- 193.5). Data presented as means +/- SEM. For full statistical details, see Appendix B.



Supplementary Figure 3: NAc KOR protein levels are upregulated in EAE after high dose U50. (A) Western blot analysis of KOR protein levels in the NAc at onset. EAE mice that received saline 1 hour prior to tissue extraction (1.164 +/- 0.084) had unchanged NAc KOR protein levels compared to CFA mice (0.934 +/- 0.092). EAE mice that received U50 (30 mg/kg, i.p.) 1 hour prior to tissue extraction (4.438 +/- 0.453) had significantly increased NAc KOR protein levels compared to CFA mice (1.087 +/- 0.050). Data presented as means +/- SEM. For full statistical details, see Appendix B.



Supplementary Figure 4: Sex differentially regulates the effect of EAE on KOR and prodynorphin mRNA in the CeA. FISH analysis of *Oprk1* and *Pdyn* mRNA in the CeA at onset. (A) In males, *Oprk1* mRNA expression is increased in EAE mice (547.5 +/- 72.8) compared to CFA mice (389.6 +/- 36.0). In females, *Oprk1* mRNA expression is decreased in EAE mice (381.2 +/- 44.0) compared to CFA mice (523.7 +/- 43.6). (B) In males, *Pdyn* mRNA expression is increased in EAE mice (1712 +/- 302) compared to CFA mice (1385 +/- 164). In females, *Pdyn* mRNA expression is decreased in EAE mice (1001 +/- 129) compared to CFA mice (1647 +/- 250). Data presented as means +/- SEM. For full statistical details, see Appendix B.

Appendix B: Table of Statistical Results

Figure	Test	Comparison	Results	P Value
1a	3way ANOVA	Time	F(1.398,43.33)=19.36	<0.0001
		Sex	F(1,31)=19.63	0.0001
		Disease	F(1,31)=73.38	<0.0001
		Time x Sex	F(9,279)=0.7330	0.6785
		Time x Disease	F(9,279)=25.25	<0.0001
		Sex x Disease	F(1,31)=1.251	0.2719
		Time x Sex x Disease	F(9,279)=3.585	0.0003
1b	2way ANOVA	Time	F(3.010,45.15)=57.81	<0.0001
		Sex	F(1,15)=15.48	0.0013
		Time x Sex	F(9,135)=4.318	<0.0001
		Subject	F(15,135)=4.913	<0.0001
1d	Unpaired t test	CFA vs EAE	t(17)=5.060	<0.0001
	2way ANOVA	Sex	F(1,15)=0.04201	0.8404
		Disease	F(1,15)=20.50	0.0004
		Sex x Disease	F(1,15)=0.05486	0.818
1e	Mann-Whitney	CFA vs EAE	U=62	0.0043
	Multiple Mann-Whitney	CFA - Males vs Females	U=28	0.175226
		EAE - Males vs Females	U=28	0.464870
2b	2way ANOVA	Treatment	F(1,6)=0.2412	0.6408
		Time	F(2.961,17.76)=40.32	<0.0001
		Treatment x Time	F(9,54)=0.1733	0.9960
		Subject	F(6,54)=3.593	0.0045
2c	2way ANOVA	Treatment	F(1,7)=0.1156	0.7438
		Time	F(1.939,13.57)=16.74	0.0002
		Treatment x Time	F(9,63)=0.4700	0.8893
		Subject	F(7,63)=6.123	<0.0001
3a	2way ANOVA	Dose	F(2.696,35.04)=23.43	<0.0001
		Sex	F(1,13)=4.317	0.0581
		Dose x Sex	F(4,52)=0.6922	0.6007
		Subject	F(13,52)=4.025	0.0002

3b	2way ANOVA	Time	F(3.863,54.09)=5.560	0.0009
		Sex	F(1,14)=9.804	0.0074
		Time x Sex	F(6,84)=1.166	0.3324
		Subject	F(14,84)=1.926	0.0346
3c	2way ANOVA	U50	F(1,14)=21.36	0.0004
		Sex	F(1,14)=3.059	0.1022
		U50 x Sex	F(1,14)=0.4982	0.4919
		Subject	F(14,14)=2.963	0.0255
	Sidak's multiple comparisons	Baseline vs U50 (Males)	t(14)=3.767	0.0042
		Baseline vs U50 (Females)	t(14)=2.769	0.0299
3d	One sample t test	Males (Theoretical mean: 0)	t(7)=3.846	0.0063
		Females (Theoretical mean: 0)	t(7)=2.656	0.0327
	Unpaired t test	Males vs Females	t(14)=0.7691	0.4546
4b	2way ANOVA	U50	F(1,32)=21.52	<0.0001
		Disease	F(1,32)=1.403	0.245
		U50 x Disease	F(1,32)=0.03209	0.859
	Tukey's multiple comparisons	Saline vs U50 (CFA)	q(32)=4.590	0.0139
		Saline vs U50 (EAE)	q(32)=4.689	0.0116
		CFA vs EAE (U50)	q(32)=1.327	0.7846
4c	2way ANOVA	U50	F(1,22)=80.40	<0.0001
		Disease	F(1,22)=0.6770	0.4194
		U50 x Disease	F(1,22)=1.410	0.2478
	Tukey's multiple comparisons	Saline vs U50 (CFA)	q(22)=11.58	<0.0001
		Saline vs U50 (EAE)	q(22)=7.012	0.0003
		CFA vs EAE (U50)	q(22)=0.3644	0.9938
4d	One sample t test	CFA (Theoretical mean: 0)	t(9)=3.348	0.0085
		EAE (Theoretical mean: 0)	t(8)=1.688	0.1300
	Unpaired t test	CFA vs EAE	t(17)=1.946	0.0684
5d	One sample t test	CFA (Theoretical mean: 900)	t(8)=4.472	0.0021
		EAE (Theoretical mean: 900)	t(9)=0.06723	0.9479
	Unpaired t test	CFA vs EAE	t(17)=2.214	0.0408
5e	Unpaired t test	CFA vs EAE	t(17)=0.5352	0.5994

6a	Unpaired t test	CFA vs EAE	t(32)=0.07477	0.9409
6b	Mann-Whitney	CFA vs EAE	U=45	0.0255
6d	Unpaired t test	CFA vs EAE	t(26)=3.513	0.0016
6e	Mann-Whitney	CFA vs EAE	U=65	0.1393
7b	Mann-Whitney	CFA vs EAE	U=51	0.0003
7d	Unpaired t test	CFA vs EAE	t(16)=0.1268	0.9007
7e	Unpaired t test	CFA vs EAE	t(16)=0.08379	0.9343
7g	Unpaired t test	CFA vs EAE	t(16)=0.7454	0.4668
7h	Unpaired t test	CFA vs EAE	t(16)=1.372	0.1891
8b	Unpaired t test	CFA vs EAE	t(35)=0.8122	0.4222
8d	Unpaired t test	CFA vs EAE	t(18)=0.1286	0.8991
8e	Unpaired t test	CFA vs EAE	t(18)=0.6517	0.5228
8g	Mann-Whitney	CFA vs EAE	U=43.50	0.9215
8h	Unpaired t test	CFA vs EAE	t(18)=1.059	0.3038
9b	Mann-Whitney	CFA vs EAE	U=28	0.5362
9d	Unpaired t test	CFA vs EAE	t(17)=0.7046	0.4906
9e	Welch's t test	CFA vs EAE	t(12.77)=2.776	0.0160
S1a	One sample t test	CFA - Males (Theoretical mean: 900)	t(4)=0.1550	0.8843
	One sample t test	EAE - Males (Theoretical mean: 900)	t(3)=4.443	0.0212
	One sample t test	CFA - Females (Theoretical mean: 900)	t(4)=4.821	0.0085
	One sample t test	EAE - Females (Theoretical mean: 900)	t(4)=0.9864	0.3798
S2a	Welch's t test	CFA vs EAE	t(25.97)=4.957	<0.0001
S2b	Unpaired t test	CFA vs EAE	t(32)=3.100	0.0040
S2c	Mann-Whitney	CFA vs EAE	U=106	0.1310
S2d	Mann-Whitney	CFA vs EAE	U=83	0.0219
S3a	2way ANOVA	U50	F(1,32)=42.39	<0.0001
		Disease	F(1,32)=46.26	<0.0001
		U50 x Disease	F(1,32)=35.15	<0.0001
S4a	2way ANOVA	Sex	F(1,16)=0.09997	0.7560
		Disease	F(1,16)=0.02272	0.8821
		Sex x Disease	F(1,16)=8.647	0.0096

S4b	2way ANOVA	Sex	F(1,16)=1.018	0.3280
		Disease	F(1,16)=0.5149	0.4834
		Sex x Disease	F(1,16)=4.804	0.0435