UNIVERSITY OF CALIFORNIA, IRVINE

Investigating Neuroinflammation and Opioid Receptor Signaling Mechanisms in Chronic Pain Pathology

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Pharmacology & Toxicology

By

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Dissertation Committee:

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GLOSSARY OF TERMS

ACC: anterior cingulate cortex

BLA: basolateral amygdala

BNST: bed nucleus of the stria terminalis

CC: corpus callosum

CCI: chronic constriction injury

CeA: central nucleus of the amygdala

CFA: Complete Freund's Adjuvant

CON: contralateral

CPA: conditioned-place aversion

CPP: conditioned-place preference

CRF: corticotropin-releasing factor

DAMGO: [D-Ala2, N-MePhe4, Gly-ol]-enkephalin

DOR: delta opioid receptor

DRN: dorsal raphe nucleus

EPM: elevated plus maze

FSCV: fast-scan cyclic voltammetry

GAD1: glutamate decarboxylase 1 (predominant isoform of GAD67)

IBA1: ionized calcium-binding adapter molecule 1

IPSI: ipsilateral

KOR: kappa opioid receptor

LH: lateral hypothalamus

MeA: medial amygdala

mPFC: medial prefrontal cortex

MOR: mu opioid receptor

NAc: nucleus accumbens

NP: neuropathic

Oprk1: kappa opioid receptor gene

Pdyn: prodynorphin gene

PFC: prefrontal cortex

PNI: peripheral nerve injury

PVH: paraventricular nucleus of the hypothalamus

TBI: traumatic brain injury

rmTBI: repeated mild traumatic brain injury

TH: tyrosine hydroxylase

VTA: ventral tegmental area

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The research underlying this dissertation was conducted by Shiwei (Steve) Liu under the auspices of Dr. Catherine Cahill and co-mentorship of Dr. Frances Leslie. Some of the behavior test experiments outlined in these studies presented in Chapter 3 and 4 were conducted by Sarah Pickens at UCI. Lihua Xue at Queen's University also contributed to behavior tests mentioned in Chapter 4. Dr. Anna Taylor spearheaded the work described in Chapter 1, as well as provided microdialysis data in Chapter 4. Acknowledgments also extend to Piper Williams of Dr. Pat Levitt's lab at the University of Southern California for their contribution of RNAscope data in Chapter 4. Dr. Jose Moron-Concepcion's lab also provided brain samples for the GTPγS data of CFA-treated rats in Chapter 4. Dr. Leslie's lab provided the remaining resources necessary for completing all GTPγS experiments described in this document.

CURRICULUM VITAE

Shiwei (Steve) Liu

EDUCATION

2013 – 2018

University of California – Irvine (UCI), School of Medicine, Pharmacology & Toxicology

- Doctor of Philosophy (Ph.D.) Candidate
- Advisor: Dr. Catherine M. Cahill, Ph.D.

2008 – 2011

The Johns Hopkins University (JHU) Krieger School of Arts and Sciences

- Bachelor of Arts – Biology
- Minor – Psychology
- Minor – Environmental Science

RESEARCH

2017 – 2018

University of California – Irvine (UCI)
Co-Advisor (Chair): Dr. Frances M. Leslie, Ph.D.
Advanced Doctorate Candidate

2014 – 2018

University of California – Irvine (UCI/UCLA)
Advisor: Dr. Catherine M. Cahill, Ph.D.
Doctoral Student

- 1 publication to a peer-reviewed journal, 2 upcoming manuscripts to be submitted
- Poster and oral presenter: UCI Pharmacology Annual Research Day
- Poster presenter: 4th Annual Cedars-Sinai Symposium
- Oral presenter: Irvine Center for Addiction Research (ICAN) Symposium
- Oral presenter: 29th Annual Spring Center for Neurobiology Learning and Memory (CNLM) Research Meeting
- Oral presenter: TED-talk AGS Symposium 2017 at UCI
- Mentored and co-mentored more than 20 undergraduate students, 4 of whom awarded the Undergraduate Research Opportunities Program (UROP) funding from UCI and another student received finalist award in the Excellence in Research Program of UCI

Dissertation: Investigating Neuroinflammation and Opioid Receptor Signaling Mechanisms in Chronic Pain Pathology

I am investigating and characterizing the aversive mechanisms of chronic neuropathic and inflammatory pain as well as repeated mild traumatic brain injury. Using the mouse model, I discovered that microglia activity is highly upregulated in brain regions that are crucial for mediating reward, emotion, and mood. In addition, these regions also showed upregulated kappa opioid receptor activation; pharmacological inhibition of these receptors attenuated rodent anxiety and depressive-like behaviors along with restoring normal dopamine neurotransmission.
.

2013 – 2014

University of California – Irvine (UCI)
Advisor: Dr. Geoffrey W. Abbott, Ph.D.
Graduate Lab Rotation Student

- 1 publication to a peer-reviewed journal
- Poster and oral presenter: UCI Pharmacology Annual Research Day
- Mentored an undergraduate student who was awarded Summer Undergraduate Research Program (SURP) funding and UROP funding

2011 – 2013

National Heart, Lung, and Blood Institute of NIH
Mentor: Dr. Vincent C. Manganiello, M.D/Ph.D.
Post-baccalaureate Intramural Scientific Research Student

- 3 publications in peer-reviewed journals
- Peer-mentored 1 graduate student and 1 post-baccalaureate student, and 4 high school students on their experiments, lab reports, and presentations for lab meetings and science conferences
- Attended the 2012 ASBMB Experimental Biology Conference at San Diego, CA
- Poster presenter: 2011 and 2012 post-baccalaureate research days at NIH

2007 – 2010 (summer)

National Heart, Lung, and Blood Institute of NIH
Mentor: Dr. Vincent C. Manganiello, M.D./Ph.D.
Summer Intern (4 total summers)

- Worked on research projects in cardiovascular and metabolism fields
- Mentored 3 high school summer interns who submitted their research project reports to national science competitions
- Presented at several annual poster days in NIH campus
- Participated in the 2008 Intel Science Talent Search, the 2008 Siemens Science Competition, and the 2008 Junior Science and Humanities Symposia

2008 – 2009

Johns Hopkins Medical Institute Pediatrics Pulmonary Branch
Supervisor: Dr. Sande Okelo, M.D.
Clinical Research Assistant

- Actively worked with physicians, nurses, and patients, documented questionnaires, vital signs, socioeconomic and demographics information
- Filed documents into hospital database for clinical meta-analysis

TEACHING/COMMUNICATION EXPERIENCE

2011

Johns Hopkins Language Lab
JHU Language Lab Staff

- Registered students into the computer lab
- Provided tutorials on language software
- Video-recorded professors' teaching, student oral presentations in archives

2011

Princeton Review Teaching Company
SAT Prep Tutor

- Taught classrooms of 15-25 high school students for Scholastic Aptitude Test (SAT)
- Proctored practice exams and grading

PUBLIC OUTREACH/VOLUNTEERING EXPERIENCE

2015 – 2017

Los Angeles Regional Brain Bee Organizer

- Volunteer to set up the Brain Bee event, a prestigious competition for high school students that tests their knowledge of neuroscience
- Writing and grading Brain Bee exams, and presenting a poster pertaining to neuroscience research at the event

2015

Diverse Educational Community and Doctoral Experience (DECADE)

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COMPUTER SKILLS FOR RESEARCH

Programming: Java, Python, Dr. Scheme, Stella Simulation & Modeling, Visual C, Interactive C

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Model organism and tissue expertise: rodent handling (mice and rats), drug injections (i.p. and s.c.), cell cultures (mammalian cell lines, stem cells, and E. coli), chronic pain model surgeries, cranial stereotaxic surgeries and cannulations, mouse organ and blood/serum sample collection, blood-perfused tissue collection, tissue biopsies

Rodent behavioral neuroscience expertise: conditioned place preference test, nestlet test, marble burying test, splash test, forced swim test, open field/locomotor test, light-dark test, elevated plus maze test, two-bottle choice tests, tail withdrawal thermal nociception test, Von Frey tactile allodynia test

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CERTIFICATIONS

2017	Nature Masterclasses Online Course Certificate
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2015	UCI Mentoring Excellence Program
2013	NIH 2013 Demystifying Medicine course – completed

2013 NIH Principles of Clinical Pharmacology course – completed

2013 Intramural Research Training Award of the National Institutes of Health

2012 NCI Translational Research in Clinical Oncology course – completed

2012 NCI Redox Biology course – completed

2012 NIH science writing and publishing course certificates

AWARDS

2017 INRC Graduate Student Travel Award ($750)

2017 SOM Outstanding Student Fellowship – UCI School of Medicine and Stanley Behrens Family Foundation ($10000, includes stipend and tuition allocation)

2017 Journal Club Best Presentation Award – UCI Pharmacology Department ($100)

2016 Public Impact Fellowship – UCI Graduate Division ($1000)

2016 ASPET Graduate Student Travel Award – Experimental Biology Conference in San Diego, CA ($850)

2016 UCI Associated Graduate Student Council Travel Grant Award – Winter Brain Conference in Breckenridge, CO ($400)

2015 UCI Travel Grant Award – NIH Career Symposium in Bethesda, MD ($500)

2011 Intramural Research Training Award of the National Institutes of Health

PROFESSIONAL SOCIETY MEMBERSHIPS

2016 Society for Neuroscience (SFN)

2015 American Society for Pharmacology and Experimental Therapeutics (ASPET)

2013 American Heart Association (AHA)

2011 American Association for the Advancement of Science (AAAS)

CONFERENCE ABSTRACTS

2017 **S. Liu**, S. Pickens, A. Taylor, L. Xue, P. Williams, J. Wu, P. Levitt, F.I. Carroll, F. Leslie, C.M. Cahill. Kappa opioid receptor upregulation contributes to mood and reward dysregulation in neuropathic pain. International Narcotics Research Conference. Chicago, IL.

2016 **S. Liu**, L. Xue, M.C. Olmstead, F. Leslie, F.I. Carroll, C.M. Cahill. Kappa Opioid Receptor Activation in Neuropathic Pain Regulates Reward. Society for Neuroscience Conference. San Diego, CA.

2016 **S. Liu**, C. Cook, E. Thai, S. Pickens, A.M. Taylor, V. Tea, F.I. Carroll, F.M. Leslie, C.J. Evans, C.M. Cahill. Neuropathic Pain Alters Reward and Affect via Kappa Opioid Receptor (KOR) Upregulation. Experimental Biology Conference. San Diego, CA.

2016 **S. Liu**, C. Cook, E. Thai, S. Pickens, A.M. Taylor, V. Tea, F.I. Carroll, F.M. Leslie, C.J. Evans, C.M. Cahill. Neuropathic Pain Alters Reward and Affect via Kappa Opioid Receptor (KOR) Upregulation. Winter Brain Conference. Breckenridge, CO.

PUBLICATIONS

2017 Kappa Opioid Receptors Drives the Tonic Aversive Component of Chronic Pain; **Shiwei (Steve) Liu**, Sarah Pickens, Anna M.W. Taylor, Hongyan Yang, Lihua Xue, Piper Williams, Chris Cook, Nicole Burma, Joshua K. Hakimian, Amie Saverino, F. Ivy Carroll, Anne M. Andrews, Mary C. Olmstead, Wendy Walwyn, Tuan Trang, Christopher J. Evans, Frances Leslie, Catherine M. Cahill. Nature Medicine.

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2013 Potential Role of Phosphodiesterase 3B in Acquisition of Brown Fat Characteristics by White Adipose Tissue in Mice; Emilia Guirguis, Steven Hockman, Oksana Gavrilova, Nalini Raghavachari, Yanqin Yang, Gang Niu, Xiaoyuan Chen, Zu Xi Yu, Faiyaz Ahmad, Youn Wook Chung, **Shiwei Liu**, Eva Degerman, and Vincent Manganiello. Endocrinology. DOI: 10.1210/en.2012-2185

ABSTRACT OF THE DISSERTATION

Investigating Neuroinflammation and Opioid Receptor Signaling Mechanisms in Chronic Pain Pathology

By

Shiwei (Steve) Liu

Doctor of Philosophy in Pharmacology & Toxicology

University of California, Irvine, 2018

Professor Frances M. Leslie, Chair

Chronic pain represents a substantial burden to society and is difficult to manage for both physicians and patients due to lack of effective therapies. There are multiple dimensions of chronic pain, with symptoms such as anxiety, depression, fatigue, anhedonia, etc. Collectively known as negative affect, these symptoms exacerbate the pain experience and results in decreased efficacy of opioid analgesics. Endotoxins known to induce neuroinflammation produce anhedonia and depression in humans. Similarly, activation of kappa opioid receptors produces dysphoric and depressive symptoms in humans. This work investigates the contribution of neuroinflammation and kappa opioid receptors in multiple rodent models of chronic pain and the manifestation of negative affect in these models. We identified that kappa opioid receptors are upregulated within limbic brain regions of neuropathic pain animals and upregulation within ventral tegmental area dopamine neurons is responsible for the tonic-aversive component of pain. This effect was sex dependent where blocking or elimination of kappa opioid receptors in male but not female mice prevented pain-induced aversion.

Our lab previously identified that neuroinflammation within the ventral tegmental area in a model of neuropathic pain was responsible for altered reward signaling and dopamine release. We show that peripheral nerve injury model of neuropathic pain causes microglial activation in many brain structures but is not ubiquitous, and neuroinflammation is primarily restricted to limbic brain structures. Because neuroinflammation is a hallmark of traumatic brain injury, we asked whether neuroinflammation is also causative to chronic pain following head injury. Using a model of repeated mild traumatic brain injury (rmTBI), we show that blocking microglial activation one month after injury reverses cold hypersensitivity and affective-like behaviors but has no effect on rmTBI-induced hyper-locomotion or risk-taking behavior. Together, we provide insight to the complexity and importance of a global neuroinflammatory response and kappa opioid receptors to pain.

CHAPTER 1: INTRODUCTION

WHAT IS PAIN?

In general, pain is a culmination of negative sensory and emotional experiences associated with potential tissue damage (Merskey & Bogduk, 1994). Pain is an important mechanism that enables one to respond to aversive, and potentially dangerous stimuli in the environment. It is a vital aspect for the body to protect itself from harm. According to the International Association for the Study of Pain (IASP), there are several types of pain sensations including: (1) allodynia is the sensation of pain to a non-painful stimulus and (2) hyperalgesia is the exaggerated response to painful stimulus. Pain can be either phasic (only during the stimulus where there is no tissue damage), short-term (acute), or long-term (chronic) (Figure 1.1). Chronic pain is defined as intermittent or constant pain lasting more than 3 months. NP pain is a subset of chronic pain defined as damage or dysfunction of the nervous system.

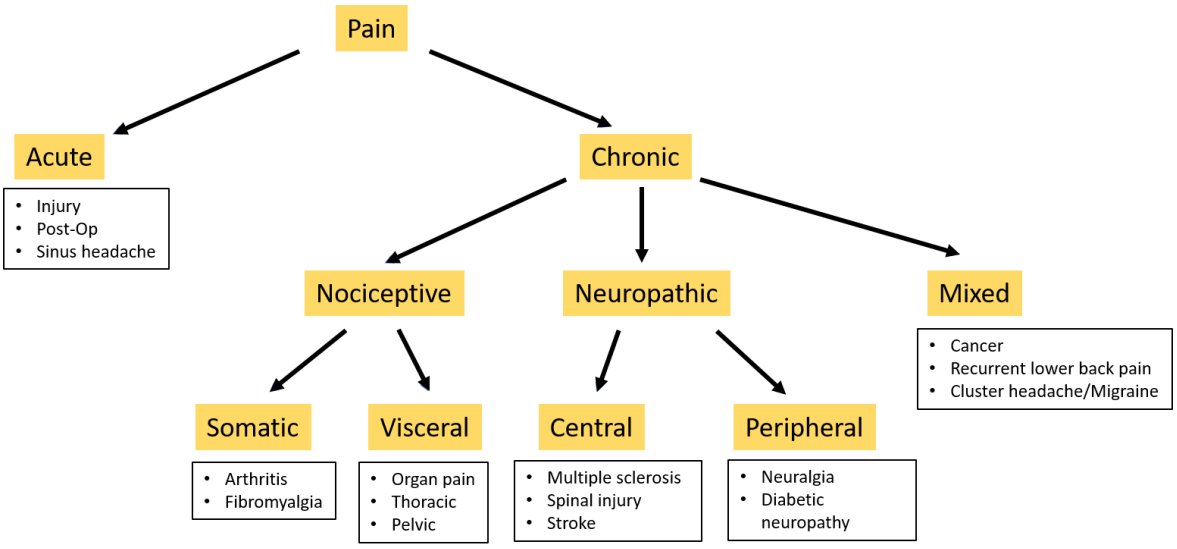

Figure 1.1 Classification of pain types. Pain is divided into acute and chronic, with further subdivisions within chronic pain. Diagram is adapted from Patapoutian, Tate, Woolf, 2009; Melnikova, 2010; Pergolizzi, Raffa, Taylor, 2014.

Pain Mechanism

Nociceptive information travels from the periphery to the ipsilateral side of the spinal cord into specific lamina within the dorsal horn. The dorsal horn contains neurons then further transmit nociceptive information to the brain by way of various fiber tracts, including the spinothalamic tract. Spinothalamic tract neurons decussate at the level of the spinal cord and ultimately reaches the contralateral thalamus to synapse with 3rd order neurons that send projections to the somatosensory cortex (Purves, et al., 2001; McMahon et al., 2013). The spinothalamic tract is primarily responsible for transmitting information about the intensity and location of pain. However, there is also a spinal projection to the parabrachial nucleus that connects with limbic structures such as the amygdala, allowing for the interpretation of the emotional, affective component of pain. Other forms of sensation such as proprioception and touch are also transmitted via primary afferents but rather than synapse in the spinal cord, the majority of these neurons project via the posterior column-medial lemniscus pathway to the medulla, where they synapse on 2nd order neurons prior to decussation and transmitting information to the brain. In response to pain stimuli, the brain responds by activating signals back to the spinal cord via the descending pathways, such as the lateral corticospinal tract that engages descending modulation pathways via the periaqueductal gray (PAG), locus coeruleus and dorsal raphe magnus that project via the dorsal lateral funiculus to the spinal cord.

Chronic Pain

Chronic pain is defined as pain perceived continuously or intermittent for a minimum of 12 weeks (National Library of Medicine, 2011), although in most cases chronic pain lasts

decades. While acute pain is an important mechanism for self-preservation, chronic pain is a pathological condition that arises from either persistent tissue damage (i.e. cancer or inflammatory diseases) or from an unknown etiology. In general, chronic pain is a serious public health problem that is a significant economic burden to society. The annual cost of both treatment and lost productivity approaches $600 billion dollars in the United States alone (Gaskin & Richard, 2012). There are approximately 1.5 billion people who suffer from chronic pain worldwide, including 25% of American population (American Academy of Pain Medicine, 2016), and chronic pain is one of the most prevalent diseases in the United States, surpassing heart disease, cancer and diabetes combined (Hall, Lee & Hruby, 2016). NP pain is one of the most challenging forms of chronic pain to treat as it is often refractory to pharmacological treatments (Gilron et al., 2006). The hallmarks of this type of pain are hyperalgesia (exaggerated response to a painful stimuli), allodynia (perception of pain to a non-painful stimulus) and spontaneous paroxysmal and burning pain. Chronic pain is highly correlated with depressive mood (Ohayon & Schatzberg, 2003). NP pain is especially highly co-morbid with mood disorders such as anxiety and depression, with correlation rates up to 80% depending on type of pain metric used (Nicholson & Verma, 2004). Treatments such as tricyclic antidepressants (TCAs) as well as serotonin and norepinephrine reuptake inhibitors (SNRIs) have traditionally also been prescribed in conjunction with opioid medication to treat NP pain. NP pain significantly and adversely impacts one's quality of life. It is imperative to develop more effective forms of chronic pain treatment.

Pain Measurement: Basic Research versus Clinic

When we mention pain relief, the most common thought is the alleviation of pain sensation. However, there are multiple facets of pain, which not only includes the sensory component, but also a cognitive component and an emotional affective component (Fernandez & Turk, 1992). Patients who suffer from chronic pain also experience memory impairment, attention deficit, and mental inflexibility for completing tasks (Krietler, 2007). Over the past several decades, methods have been attempted to measure pain and pain relief in the clinic (Lasagna, 1960). Many commonly used pain scales involve a form of questionnaire survey (Melzack, 1975; Stratford, et al., 1998; Hicks, et al., 2001). These clinical pain scales do not solely reflect one's sensory component of pain, but rather the processing of pain at both a cognitive and emotional level. The patients are asked to rate their pain, which is not a direct measurement of pain thresholds but incorporates all the dimensions of pain. However, these methods cannot be used in non-primate animals for basic research. Although there are models that can test for different aspects of negative affect such as anxiety and depressive-like behaviors in animals, the majority of basic pain research focuses on quantifying sensory pain levels based on tactile, thermal, and chemical nociception thresholds (Mogil, Wilson, & Wan, 2001), pain response behavior such as avoiding limb motions that can cause pain (Duncan, et al., 1991; Gabriel, et al., 2007), or animal vital signs such as weight loss (Liles & Flecknell, 1992; Clark, et al., 2004; Traul, et al., 2015). To capture the affective component of pain, basic research has started borrowing methods used traditionally for addiction and mood disorder research. The conditioned place preference paradigm used to study rewarding effects of drugs has now been validated as an effective measure for the tonic-aversive component of pain (Cahill et al., 2014). That is, analgesic drugs that have no rewarding effect on their own will produce a place preference in chronic pain animals. For example, peripheral nerve blocks with lidocaine will

produce a place preference in animals experiencing pain but not in pain-naïve animals (Navratilova, et al., 2012). This effect is referred to as negative reinforcement because the reward is due to removing a negative state rather than reward producing a hedonic positive effect. Negative affect caused by pain can result in anxiety, depression, and decline in motivation (Gaskin, et al., 1992; Craske, et al., 2009; Auvrey, Myin & Spence, 2010). Pain researchers use behavioral testing paradigms that have been used to screen effectiveness of anti-anxiety and anti-depressant drugs, which have been able to capture the negative affect in animals with chronic pain (Ohl, 2005; Castagne, et al., 2011).

Current Chronic Neuropathic Pain Treatments

A common treatment for chronic pain involves pharmacological approaches with oral analgesics. These can be traditional analgesics such as acetaminophen, opioids, non-steroidal anti-inflammatory drugs (NSAIDs) or non-traditional drugs, which include anticonvulsants and antidepressant drugs that modulate nociceptive transmission (Melnikova, 2010). Opioid analgesics are recommended for the treatment of moderate to severe pain by the World Health Organization; although opioids are very effective for treating acute post-operative and trauma-induced pain, their effectiveness in alleviating non-cancer chronic pain is the subject of much debate (Turk, Wilson & Cahana, 2011; Jamison & Mao, 2015). Opioids produce analgesic effects throughout the central nervous system. In the spinal cord, they act at presynaptic terminals of primary afferent nociceptors, causing a blockade of voltage-gated calcium channels that prevent further pain signaling via blocking neurotransmitter release, as well as act post-synaptically to enhance activity of voltage-gated potassium channels on pain transmission

neurons that leads to hyperpolarization to reduce neurotransmission (Werz & Macdonald, 1982; Macdonald & Werz, 1986; Morgan & Christie, 2011; Williams, et al., 2013). The use of opioids in pain therapy has been steadily becoming more prevalent over time (Alam & Juurlink, 2016). Sales of prescription medications have skyrocketed with an increase in retail sales of 1,293% for methadone, 866% for oxycodone, and 525% for fentanyl from 1997-2007 (Manchikanti, et al., 2010). Similarly, the increase in therapeutic opioid use in the United States in milligrams per person from 1997 to 2007 increased 402% overall (Manchikanti, et al., 2010). Further, Americans consume 99% of the world use of hydrocodone. However, opioids carry a grave side effect as users can become addicted to these medications after prolonged use (Juurlink & Dhalla, 2012; Webster, 2012; Cicero & Ellis, 2015a; Cicero & Ellis, 2015b; Cicero & Ellis, 2015c, Cicero, Ellis & Harney, 2015). These can lead to increased risk of overdose and death, which has doubled from 1996 to 2007 (Centers for Disease Control, 2009); this rate continues to increase annually as more drugs and higher dosages are being prescribed. Overdose occurs with both prescription opioids such as morphine and oxycodone as well as heroin, which on the streets is considerably cheaper; approximately 75% of patients with prescription opioid drug addiction switch to heroin as a cheaper opioid source (Cicero, Ellis & Harney, 2015). In essence, opioid abuse is rampant in the US with over 37% of all drug overdose deaths related to prescription opioids in 2013 (American Society of Addiction Medicine, 2016).

Neuroinflammation in Chronic Pain

Neuroinflammation is causative to the development of chronic pain. It has been demonstrated previously that microglia and astrocytes in the nervous system are activated during

chronic pain, releasing pro-inflammatory factors (i.e. TNF-α and IL-1β) that alter sensory neuron signaling and contribute to allodynia and hyperalgesia (Tsuda et al., 2003; Raghavendra et al., 2004). Accordingly, inhibiting the pro-inflammatory glial cells may be an effective treatment for chronic pain. However, this strategy is not so simple, as clinical studies on glial inhibitor analgesics have been unsuccessful (Martinez et al., 2013; Vanelderen et al., 2015; Sumitani et al., 2016). Furthermore, some other anti-inflammatory therapeutics can even become toxic to nervous tissue (Yurt and Kaplan, 2018). Therefore, we still need to understand the complexities of neuroinflammation in chronic pain pathology.

Many signaling networks play a role in the overall neuroinflammation phenotype of chronic pain. For example, mast cells contribute to cytokine signaling and development of new pro-inflammatory cells that interact with glia in the nervous system (Skaper et al., 2017). Other studies have attempted to examine the interactions between astrocytes and microglia, which together may drive pro-inflammatory signaling more profoundly than microglia alone (Facci, Barbierato, and Skaper, 2018). Neuroinflammation is also influenced by inflammatory signals systemically as well. Monocyte migration to the central nervous system is enhanced during chronic pain, which may interact with the dorsal root ganglia sensory signaling as well as glial cells in the spinal cord, ultimately shaping pain transmission (Huh, Ji, and Chen, 2017). Pro-inflammatory macrophages at peripheral sites of injury can also release cytokines and chemokines that drive pain hypersensitivity (Kiguchi et al., 2017). Moreover, these macrophages carry inflammasome signaling, a secondary mechanism that can trigger NP pain states in rodents (Khan et al., 2018). Due to the many inflammatory mechanisms that affect chronic pain pathology, it is necessary to further elucidate these pathways for the development of effective therapeutics.

Chronic Pain from Traumatic Brain Injury

The rate of traumatic brain injury (TBI) is estimated to be at least 106 per 100,000 people worldwide (Hyder et al., 2007), with mild traumatic brain injury (mTBI) potentially even more prevalent (upwards of 600 per 100,000 people) due to such injuries going unreported (Cassidy et al., 2004). Chronic pain is common in TBI patients (Irvine and Clark, 2017) with an occurrence rate of between 43-58% (Nampiaparampil, 2008). Chronic headache is an extremely prevalent outcome after TBI (Defrin, 2014). One potential mechanism for this may be the plasticity and reorganization of pain signaling neurons that occur after an injury (Kuner and Flor, 2016). This plasticity may lead to alterations of descending noxious inhibitory control that produces an overall sensitization of pain signals (Arendt-Nielsen et al., 2010; Boyer et al., 2014). There has also been evidence to suggest that TBI can trigger synaptic restructuring in critical sensory processing regions such as the hindbrain (i.e. PAG and medulla) and the thalamocortical system that may be responsible for chronic migraines and generalized pain hyperalgesia (Defrin et al., 2015). Neuroinflammation may also play an impactful role in disrupting pain signaling following TBI (Irvine et al., 2018), which can result in chronic pain.

Since chronic pain has steadily become a hot topic in the TBI research field, much of the behavioral and mood dysregulations that stem from TBI need more exploration, especially in mTBI (Grandhi et al., 2017). Mild TBI can cause irritability, anxiety, depression, and drastic motor and cognitive impairment for patients (Dischinger et al., 2009; De Beaumont et al., 2011; Ponsford et al., 2012; McInnes et al., 2017). These are common symptoms that also arise from chronic pain, which makes understanding TBI mechanisms also helpful to chronic pain research.

Today, treating patients with TBI is an extremely challenging task because of the diverse pool of negative symptoms, both physical and psychological. Current therapies, while effective in many cases, typically do not have total coverage of the multiple dimensions of TBI (and chronic pain) pathology. Therefore, it is imperative to study the mechanisms underlying all of these symptoms following TBI. This is especially necessary for treating mTBI since the prevalence of this injury is likely far greater than is represented in clinical reports.

Implications of Dopamine Signaling in Chronic Pain

Dopamine levels and evoked dopamine release in the nucleus accumbens is proposed to be linked to pain analgesia, motivation, and drug addiction (Baliki, et al., 2010; Sarkis, et al., 2011; Apkarian, Baliki & Farmer, 2013; Schifirnet, Bowen & Borszcz, 2014; Navratilova, Atcherley & Porreca, 2015; Taylor, et al., 2016). One of the major dopamine signaling circuitries involve the mesolimbic reward pathway (Figure 1.2). Dopamine cell bodies located in the ventral tegmental area (VTA) project to other regions such as the nucleus accumbens (NAc), prefrontal cortex (PFC) (Seamans and Yang, 2004), cingulate gyrus (Richter and Gruber, 2018), hippocampus (Nobili et al., 2017), and amygdala (Fudge et al., 2017). The topography of the VTA is neither uniform nor homogeneous; dopamine neurons in this region are not evenly scattered, but rather spatially positioned in a specific way designed to organize their projections to other regions. Projections from the VTA do not send collaterals to other regions (Beier et al., 2015). In addition, there are other cell types within this region that regulate dopamine neurons and modulate the release of dopamine. GABA neurons are present in the VTA, and form synapses that regulate each other (Allison et al., 2006) as well as provide inhibitory input to

dopamine neurons (Simmons, Petko, and Paladini, 2017). These various interactions in the VTA all significantly contribute to the plasticity mechanisms of the reward pathway.

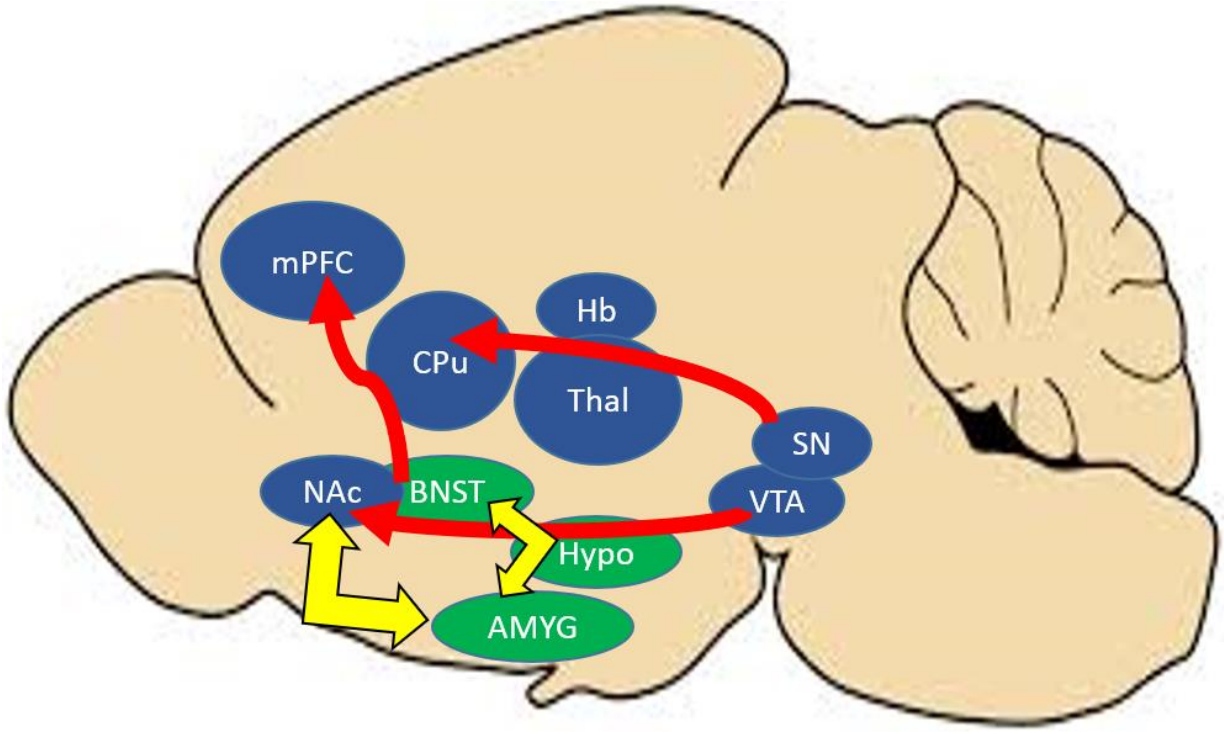

Figure 1.2. Simplified diagram of reward, motivation, and stress pathways. All regions depicted are involved in regulating mood and reward. These include the medial prefrontal cortex (mPFC), caudate putamen (CPu), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), habenula (Hb), thalamus (Thal), hypothalamus (Hypo), amygdala (AMYG), ventral tegmental area (VTA), and substantia nigra (SN). Red arrows indicate dopaminergic signaling networks across brain regions in blue. Yellow arrows indicate signaling among brain regions in green that are involved in stress response.

It has been proposed that TBI causes impairment of dopamine signaling in the brain (Bales et al., 2009), a possible source of chronic pain in TBI patients. Pain induced by migraines has been linked to various brain regions within the reward pathway, such as the NAc, anterior cingulate cortex (ACC), and the amygdala (Cahill, Cook & Pickens, 2014). Animals can also have compensatory mechanisms to positively cope with the unpleasant experience, which causes shifts in mesolimbic circuits that may enhance dopamine and serotonin release (Sagheddu, et al., 2015). However, during chronic pain, there is significantly reduced dopamine signaling in the brain (Martikainen, et al., 2015; Dieb, et al., 2016; Taylor, et al., 2016). Dopamine precursor depletion can greatly influence pain affect rather than pain perception (Tiemann, et al., 2014). Furthermore, patients with schizophrenia exhibit abnormally high amounts of basal dopamine levels in the brain, are correlated with pain hyposensitivity (Jarcho, et al., 2012). During chronic pain, reduced dopamine tone may play a significant role in the negative affective component of pain. Indeed, microglia in the VTA release brain-derived neurotrophic factor that heightens activation of GABAergic interneurons acting to inhibit dopaminergic neurons (Taylor, et al., 2015a). Dopamine can modulate the function of the amygdala. In particular, polymorphisms of dopamine transporter genes can alter amygdala processing of aversive emotional stimuli (Bergman, et al., 2014). Thermal pain aversion is also highly regulated by the NAc and ACC (Becerra, et al., 2013), and dopamine neurons projecting to the NAc are thought to be involved in reward, whereas those projecting to the PFC are associated with aversion (Lammel, et al., 2011).

OPIOID RECEPTORS

History

Opium has been known for thousands of years as a compound that could alleviate many ailments. It was known to be a pain killer and had euphoric properties, implicating many geopolitical events such as wars throughout history. Only within the last 200 years have the mechanisms of this compound and its derivatives have been carefully studied. Since then, further discoveries of endogenous opioid ligands as well as cloning of the targeted opioid receptors have provided us more knowledge of their signaling in the body. During the 1960s and 1970s, studies on various analgesics like morphine provided insight into possible opioid receptors within the body that could regulate pain signaling. Eventually, three classes of opioid receptors were identified, MOR, DOR, and KOR (Martin, 1979) and more recently, the nociceptin receptor (NOP) has been added to the opioid receptor family. All of these opioid receptor subclasses are able to form dimers and heteromers with each other, or with other types of G protein coupled receptors, vastly diversifying their signaling roles in the cell (Jordan et al., 2001; Van Rijn, Whistler & Waldhoer, 2010; O'Dowd et al., 2012; Ong and Cahill, 2014; Akgun et al., 2015; Moreno et al., 2017).

Opioid Receptor Ligands

The original notion of opioid receptor function was to mediate the effects of opium alkaloids used in medicine. However, this thought process seemed imperfect, as the body should naturally be utilizing these receptors for specific roles. By the 1970s, researchers began to postulate that the body produces endogenous ligands that could activate these opioid receptors. Eventually, two enkephalin ligands were isolated in pig brain (Hughes et al., 1975). A third ligand, dynorphin, was characterized a few years later (Goldstein et al., 1981; Chavkin &

Goldstein, 1981). Shortly thereafter, these ligands were shown to bind to specific opioid receptor classes (Paterson et al., 1984).

IMPORTANCE OF KAPPA OPIOID RECEPTORS IN PAIN

Kappa Opioid Receptors and Dopamine

There is a wealth of literature on chronic drug abuse and withdrawal. One of the prominent features of kappa opioid receptor (KOR) activation is its ability to cause anxiety and depressive-like symptoms in humans and rodents (Land, et al., 2008; Van't Veer & Carlezon, 2013; Hang, et al., 2015). KOR agonists are well-known to cause dysphoria and psychotomimesis (Pfeiffer, et al., 1986), and drug conditioned place aversion in rodents (Tajeda, et al., 2013). Furthermore, KORs have been implicated in negative affect symptoms of withdrawal from many drugs of abuse, such as in cocaine (Chartoff et al., 2012), ethanol (Siciliano, et al., 2015), nicotine (Sudakov, et al., 2014), methamphetamine (Whittfield, et al., 2015), and heroin (Sedki, et al., 2015). All of these drugs of abuse manipulate the dopamine-based circuits in the brain and can lead to dopamine signaling deficits with prolonged use. One proposed mechanism for chronic drug-induced negative affect symptoms is chronic drug intake causing dynorphin release in the striatum, which signals KORs in this region to reduce further dopamine release as a homeostatic mechanism (Bruijnzeel, 2009). Cessation of drug intake does not recover the reduced basal dopaminergic tone, which potentially contributes to the persistent negative emotional state during drug withdrawal that causes a drug abuser to maintain further drug use. Thus, KOR activation corresponds to global inhibition of dopamine signaling in the mesolimbic system including, but not limited to, the PFC, NAc, and VTA (Lalanne, et al., 2014).

As described in the previous section, dopamine signaling is heavily implicated in pain pathways, with this neurotransmitter being critical for pain analgesia. Interestingly, KOR expression is present in brain regions that are involved in *both* drug withdrawal (Sun, 2011) and pain circuits (Taylor, 2013; Cahill, et al., 2014). The cognitive and emotional states observed in drug addiction and withdrawal studies are highly similar to those in the chronic pain state, such as anxiety, depression, dysphoria, and anhedonia (Nicholson & Verma; O'Connor, 2009; Elman, Borsook & Volkow, 2013). These similarities may not be a coincidence, and perhaps KOR signaling is important in not only drug addiction and withdrawal, but also in negative affect during chronic pain.

Kappa Opioid Analgesics

Like all opioid receptors, KOR activation has been known to produce antinociception by regulating Ca^{2+} channels (Hassan & Ruiz-Velasco, 2013). In addition, activating KORs using the highly-selective agonist U50,488 can have antinociceptive effects (Koob, et al., 1986; Barr, Wang & Carden, 1994; McLaughlin, et al., 2006; Land, et al., 2008; Land, et al., 2009; Robles, et al., 2014). While activation of KORs within the spinal cord can produce antinociception by reducing pro-nociceptive neurotransmitter release, systemic KOR agonist administration appears to produce antinociception that is due to activation of stress circuitry. Specifically, the antinocicpetion resulting from systemic administration of KOR agonists can be blocked by anxiolytics such as benzodiazepines or DOR agonists (Taylor et al., 2015). KOR signaling for antinociception is induced through either a JNK-mediated pathway with endogenous ligand, or ERK-mediated pathway with a synthetic agonist such as U50,488 (Jamshidi, et al., 2015). On the

other hand, activating KORs with highly specific agonists such as U50,488 or U69,593 is aversive and can lead to dysphoria (Land, et al., 2008; Tajeda, et al., 2013). Due to the dysphoric effects induced by KOR agonists, analgesics targeting this receptor have largely been unfruitful. Only very recently has there been a better understanding of KOR signaling mechanisms, by which analgesia and dysphoria are in fact mediated via independent signaling cascades from the initial activation of KOR (Ehrich, et al., 2015). Drugs such as nalfurafine and noribogaine have been developed for KOR that are potentially non-addictive biased agonists focusing on the analgesic component involving ERK, and not the β-arrestin signaling that causes dysphoria (Nagase & Fujii, 2013; Maillet, et al., 2015; Nakao, et al., 2016). However, further research is needed to confirm that these types of drugs are safe for widespread clinical use. On the other hand, my preliminary studies on chronic NP pain outlined in this thesis suggest that KOR activation is upregulated in the brain of mice with chronic NP pain. My thesis aims to understand how chronic pain alters the expression and function of the KOR, as well as the extent to which KORs contribute to the aversive dysphoric effects associated with chronic pain, and to provide further validation of pursuing KOR antagonists as a therapeutic target for chronic pain.

Sex-Dependent Opioid-Based Analgesic Efficacy

Clinical research has shown that men and women experience different pain thresholds, whereby females are more sensitive to noxious stimuli (Riley, et al., 1998; Mogil & Bailey, 2010). It is generally recognized that men have higher pain tolerance thresholds than women, and typically require less anesthetics when undergoing painful surgical operations (Goolkasian, 1985; Hussain, et al., 2013). Part of the reason for differences in pain perception can be

attributed to the sexual dimorphism of brain regions, such as the hypothalamus and bed nucleus of the stria terminalis (BNST) (Swaab & Fliers, 1985; Allen & Gorski, 1990). Opioid receptors are also involved in modulating pain perception and may explain some of the sex differences seen in pain reporting. It has been shown that mu-opioid receptors (MORs) form heteromers with KORs at the spinal cord level (Liu, von Gizychi & Gintzler, 2007) that may modulate morphine or fentanyl antinociception as well as altering their side effects (Constantino et al., 2012; Fujita, Gomes, and Devi, 2015). These heteromers are found in high abundance in female rats during certain periods of their estrous cycle (Chakrabarti, Liu & Gintzler, 2010). In addition, at their peak abundance, there are roughly four times more MOR/KOR heteromers in females than in males, which could contribute to sex differences in pain perception. A prior study outlined the MOR activation profile in brains of women during the follicular phase of their menstrual cycle compared to MOR activation in male brains; they found striking differences of receptor activation in multiple brain regions (Zubieta, et al., 2002). Men had higher activations of MOR in the thalamus, ventral basal ganglia, and amygdala, and women had even lower basal MOR activity levels than men in their NAc. From the notion gleaned from studying MOR in pain perception, it is therefore plausible to assume that other opioid receptors such as KOR would also be differentially activated in various brain regions depending on sex of the individual. This assumption is supported by the fact that estrogen is heavily linked to dynorphin signaling in the female rodent brain (Fullerton, Smith & Funder, 1988; Torres-Reveron, et al., 2009; Gottsch, et al., 2009; Mostari, et al., 2013). Estrogen can increase KOR gene expression in both female rodents and humans at the spinal cord level (Dawson-Basoa & Gintzler, 1996). Estrogen has also been shown to increase KOR expression in the brain, thereby enhancing KOR-mediated anti-nociception and anti-hyperalgesia in rats (Lawson, et al., 2010). This anti-nociceptive effect can

further be enhanced with U50,488, a KOR-specific agonist, but can be eliminated by ovariectomy. However. in male rats there was no significant link between testosterone and KOR-mediated antinociception, which suggested KOR involvement in antinociception is sex-dependent. On the clinical front, studies have confirmed that using KOR agonists as analgesics are more effective in women, than in men (Gear, et al., 1996a; Gear, et al., 1996b; Gear, et al., 1999), which may be due to fewer aversive side effects, such as dysphoria, experienced in women. Another possible mechanism for differences in KOR-mediated analgesia due to sex could be that women express melanocortin 1 receptor (MC1R) variant alleles that are different from MC1R alleles in men; this gene mediates KOR analgesia in female mice and not male mice, which is actually positively correlated to findings in humans (Mogil, et al., 2003). A summary of animal studies using various KOR agonists in a variety of experimental conditions and animal strains across sexes (Craft, 2003). The conclusions made from the reviewed studies were conflicting, and these inconsistencies have been reported in later studies as well. For example, one study showed that KOR-induced analgesia to inflammatory pain was greater in male versus female rodents (Lomas, et al., 2007). This notion was further updated in more recent reviews (Dahan, et al., 2008; Mogil, 2012). Unfortunately, this observation does not reflect what is observed in clinical studies, as certain KOR-based analgesics such as nalbuphine produce greater pain relief in women than in men, and women have fewer negative affect-like side effects than men (Gear, et al., 1999; Gear, et al., 2003; Kshirsagar, et al., 2008). Our lab has also previously confirmed sex differences in pain perception through KOR activation by isolating its analgesic effects using MOR-knockout mice treated with the highly-selective delta opioid receptor (DOR) antagonist, naltrindole (Taylor, et al., 2015b). From this model, it was demonstrated that KOR activation could induce robust antinociception in male mice but less

antinociception in female mice. This effect was stress-induced and was blocked by benzodiazepine and DOR-mediated anxiolytics. Thus, based on the diverse literature studying KOR-based analgesia in males and females, as well as rodents and humans, it is highly evident that our knowledge of the pathways involved in these systems is not complete. Yet, it is imperative to understand these differences in order to effectively treat chronic pain in both sexes.

RESEARCH RATIONALE AND OBJECTIVES

Chronic pain is a significant burden worldwide, with current treatments being unable to address the multifaceted components of pain, which include negative affect. Chronic pain in the form of recurrent headaches resulting from brain injury also negatively impact's patient quality of life. Use of opioid-based therapeutics is prevalent in today's society; however, their usage also has drawbacks. **The objective of my research is to determine the mechanisms of neuroinflammation that alter brain circuitry, as well as the involvement of the kappa opioid receptor in these pathways during chronic pain.** I investigated microglial activation in various brain regions of rodents with NP pain, as well as their function after repeated mild TBI. I also sought to study changes in KOR expression and function in the brain of animals with either chronic NP or inflammatory pain, as well as whether sex differences were involved, because chronic pain perception and adaptation are different in women and men.

Global Hypothesis

Chronic pain and repeated brain injury result in upregulation of microglial and kappa opioid receptor activity that alter mood and affect, which ultimately exacerbate the pain experience.

Specific Aim 1

Neuroinflammation in chronic pain has recently been shown to alter chronic pain pathology. While there is abundant evidence that neuroinflammation via microglial reactivity at the level of the spinal cord contributes to pain hypersensitivities, it remains unknown whether neuroinflammation occurs in the brain during chronic pain states. I asked whether microglial

reactivity occurs in the mesolimbic circuitry of chronic pain animals and the extent in which microglial reactivity modulates nociception, dopamine levels, and opioid-induced effects.

Specific Aim 2

Repeated mild TBI has been showed to induce neuroinflammation that may contribute to brain plasticity recovery mechanisms. However, repeated brain injury can impair normal brain function such as cognition, lead to migraines, and is associated with anxiety and depression. I asked to what extent microglial reactivity caused by brain injury alters pain sensitivity, mood, and synaptic restructuring in the brain.

Specific Aim 3

KOR agonists have been known to produce antinociception and dysphoric effects including depressive-like behaviors. Thus, I aimed to determine the extent KOR expression and function may be altered in chronic pain states and whether KOR contributes to the negative affect precipitated by chronic pain. I also investigated differences in regional KOR activation between male and female NP pain mice in order to elucidate potential sexually dimorphic mechanisms involving pain. Our lab has previously shown that opioid- and cocaine-evoked dopamine release is blunted in NP pain and that reward behavior produced by these drugs is also blunted in NP pain (Taylor et al., 2015). It is known that activation of KORs can modulate dopamine release. Thus, I administered KOR antagonists into mice to assess the precise involvement of KORs that may modulate reward in NP pain animals. I determined the occurrence of anxiety and depressive-like behaviors in mice with NP pain and whether these behaviors are dependent upon KOR activation.

CHAPTER 2: TOPOGRAPHY OF MICROGLIAL ACTIVATION IN SENSORY- AND AFFECT-RELATED BRAIN REGIONS IN CHRONIC PAIN

Introduction

Prior studies on chronic pain have shown there is significant upregulation of neuroinflammatory markers, such as cytokines and chemokines (Ellis and Bennett, 2013; Ji, Xu, and Gao, 2014; Huh, Ji, and Chen, 2017). At the site of the injury, modifications of sensory neurons occur through the signaling of substance P and brain-derived neurotrophic factor (BDNF), which alter their firing potential (Traub, 1996; Kerr et al., 1999). In addition, microglia directly change the excitability of lamina I neurons by disrupting their anion gradients (Coull et al., 2003), which disinhibits them and causes them to fire more readily. This mechanism was shown in neuropathic pain; ATP-stimulated microglia was demonstrated to release BDNF and caused a collapse in the anion gradient of these sensory neurons, which contributed to tactile allodynia (Coull et al., 2005). Therefore, the inflammatory response in chronic pain may be a significant contributor to the maladaptive changes in the brain that exacerbate the pain experience.

Neuroinflammation also occurs in the brain during chronic pain. Glial cells have been shown to be highly activated in patients with chronic back pain (Loggia, et al., 2015). Mood disorders are highly associated with chronic pain (Nicholson and Verma, 2004; Juurlink et al., 2004; Asmundson and Katz, 2009) and neuroinflammation (Pfau, Menard and Russo, 2017). Interestingly, past studies have shown that neuroinflammation in the brain may inhibit the

effectiveness of antidepressant medication (O'Brien et al., 2007; Carvalho et al., 2013; Uher et al., 2014). In an effort to determine the causal link between these phenomena, our lab identified microglia upregulation in response to chronic NP pain led to reduced dopamine transmission in the rodent midbrain, which is a mechanism contributing to mood disorders (Taylor et al., 2015). In this chapter, I describe a follow-up study conducted to more thoroughly assess the topographical extent of microglial involvement in other brain regions during NP pain.

Methods

Animals

Thirty-four male, 8–10-week-old C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in groups of four on a 12-hr light/dark cycle, with food and water available ad libitum. No animals were excluded from analysis in any of the behavioral or molecular assays. All procedures were conducted in accordance with the National Institutes of Health Guide for the care and Use of Laboratory Animals for Research (ULAR) and were approved by the University of California, Los Angeles, and the University of California, Irvine, Institutional Animal Care and Use Committees (IACUC).

Peripheral Nerve Injury

Animals were randomly assigned to one of two surgery groups, sham or peripheral nerve injury (PNI). Surgery was performed as described previously (Mosconi and Kruger, 1996; Taylor et al., 2015). All animals were anesthetized with gaseous isoflurane (induction at 5% and maintenance at 2.5% in oxygen). For the peripheral nerve injury (PNI), an incision was made in the lateral left thigh, followed by a blunt dissection of the left lateral thigh to expose the sciatic

nerve. The nerve was encased with a polyethylene tube (PE20, 2 mm long). The control group (sham surgery) received a similar surgery without dissection of the nerve. Animals were allowed to recover in their home cages for 2 weeks.

Behavior

Two weeks after nerve injury, mice were placed in a 10 × 10-cm Plexiglas box on a wire mesh floor (n = 6 per group, based on previous experience with this technique; Taylor et al., 2015). After 30 min of acclimation to the environment, mechanical thresholds were assessed in the ipsilateral (left) paw of sham and PNI animals. Von Frey filaments of incremental stiffness were applied, and a positive reaction was recorded after vigorous retraction of the hind paw. The up-and-down method (Dixon, 1991) was used to measure the 50% withdrawal threshold (Chaplan et al., 1994).

Immunocytochemistry

To assess PNI-induced microglial activation, tissue was processed for immunocytochemistry 2 weeks after nerve injury surgery. This time point was selected because we had previously shown that it correlates with peak pain behavior and robust microglial activation in the VTA (Taylor et al., 2014). Animals (n = 6 per group) were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was dissected from the skull and post-fixed in 30% sucrose before being cryo-sectioned at 50 μm. Whole brains were divided into left and right hemispheres, and coronal sections were collected in 0.1 M phosphate-buffered saline (PBS) with 0.2% Triton X-100 (PBS-T) and incubated with blocking buffer (5% normal goat serum) for 1 hr prior to being immunolabeled for microglia with an anti-ionized

calcium-binding adaptor molecule 1 (Iba-1) antibody (1:2,000; rabbit polyclonal; RRID:AB_839506; catalog No. 016-20001; Wako, Osaka, Japan; previously validated; Imai et al., 1996). Labeling was visualized with a goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1,000; Invitrogen, Carlsbad, CA). For analysis, images were acquired on an Olympus (Tokyo, Japan) BX51 fluorescent microscope with a 20x objective. Microglial activation was quantified by measuring cell body size in ImageJ. This technique has been validated against our previous methods with defined qualitative criteria to assess activation (Kozlowski and Weimer, 2012; Taylor et al., 2015, 2016). At least 20 cells over three separate slices per region per animal were measured and averaged to give a single value per animal. An experimenter blind to conditions performed the imaging and the quantification.

Quantitative Real-Time PCR

Brains were collected from sham and PNI mice (n=5/group) 8 weeks post-surgery, coronal sectioned via cryostat (150-µm thick) at −20°C, and mounted on charged Superfrost slides. Tissue punches (1 mm diameter) were taken by using a disposable biopsy plunger (Miltex, York, PA) for medial prefrontal cortex (mPFC), nucleus accumbens (NAc), bed nucleus stria terminalis (BNST), amygdala (AMYG), hippocampus (HIPP), thalamus (THAL), VTA, and dorsal raphe nucleus (DRN). Because the nerve injury was performed in only the left hind leg of the mouse, brain tissue regions from the left and right hemispheres were assessed separately.

Total RNA was collected from the brain tissue punches via Trizol extraction method (Ambion Life Technologies, Grand Island, NY). RNA was converted to cDNA with 100 U M-MulV reverse transcriptase, 1 µM oligo d(T)23VN, and 2 mM dNTP mix (New England Biolabs, Ipswich, MA); annealed at 70°C; and inactivated at 95°C. Quantitative real-time PCR (qRT-

PCR) was conducted with primer sets for Iba-1 (with primers for Iba1 Iba1-F, ATC AAC AAG CAA TTC CTC GAT GA; Iba1-R, CAG CAT TCG CTT CAA GGA CAT A) and β-actin (with primers for actb as a control gene Actb-F, GGC TGT ATT CCC CTC CAT CG; Actb-R, CCA GTT GGT AAC AAT GCC ATG T). With 96-well optical plates (Applied Biosystems, Singapore), cDNA and PerfeCTa SYBR Green FastMix containing the primer sets (Quanta Biosciences, Gaithersburg, MD) were loaded and run on an ABI ViiA7 fast block qPCR machine with cycling conditions from the PerfeCTa SYBR Green FastMix manual. Cycle threshold outputs were calculated and normalized to the actin housekeeping gene to compute ΔCt. Relative expression levels were determined by normalizing sham and PNI groups to actin control via the ΔCt method. The results expressed as relative expression (2ΔCt).

Statistics and Data Analysis

For behavioral data, withdrawal thresholds were averaged among groups and compared by Mann-Whitney test, and significance was set at $P < 0.05$. For immunohistochemistry, data are expressed as means +/- SEM per group and compared by two-way ANOVA, followed by Bonferroni post hoc analysis for comparing surgery and side. Differences were considered statistically different at $P < 0.05$. For quantitative RT-PCR, data are expressed as means +/- SEM and analyzed by one-way ANOVA, with Bonferroni correction between sham and PNI brain regions. $P < 0.05$ was considered significant.

Results

PNI mice at 2-weeks post-surgery displayed increased tactile allodynia compared to sham counterparts (sham 0.91 g ± 0.10 versus PNI 0.14 g ± 0.04, $p < 0.01$). Brains were collected from

these mice at the 2-week time point and had significantly increased Iba1 gene expression in the contralateral hemisphere of the brain (Table 2.1). These results are consistent with hypothesis that chronic pain induces neuroinflammation due to pain signals at the ipsilateral surgery side traveling to the spinal cord and decussating before reaching to the contralateral side of the brain. In particular, expression of Iba1 in the NAc, thalamus, and VTA was significantly increased in PNI animals compared to sham counterparts (Figure 2.1). These results overall show that microglia gene expression in the brain is indeed increased in NP chronic pain, and alludes to their responsive type of role towards pain signaling.

	Sham		**PNI**	
Brain Region	**Ipsilateral**	**Contralateral**	**Ipsilateral**	**Contralateral**
mPFC	0.13 (0.07)	0.06 (0.03)	0.12 (0.12)	0.17 (0.10)
NAc	0.15 (0.04)	0.32 (0.03	0.23 (0.07)	0.62 (0.03)*
BNST	0.14 (0.08)	0.14 (0.05)	0.42 (0.02)	0.74 (0.06)*
Amygdala	0.62 (0.05)	0.54 (0.06)	0.71 (0.04)	1.21 (0.07)
Hippocampus	0.31 (0.19)	0.36 (0.02)	0.15 (0.05)	0.12 (0.06)
Thalamus	0.35 (0.07)	0.28 (0.05)	0.47 (0.07)	0.74 (0.04)*
VTA	0.14 (0.14)	0.19 (0.07)	0.42 (0.11)	0.67 (0.08)

Table 2.1. Iba-1 gene expression in brain regions between sham and PNI mice. *$p<0.05$

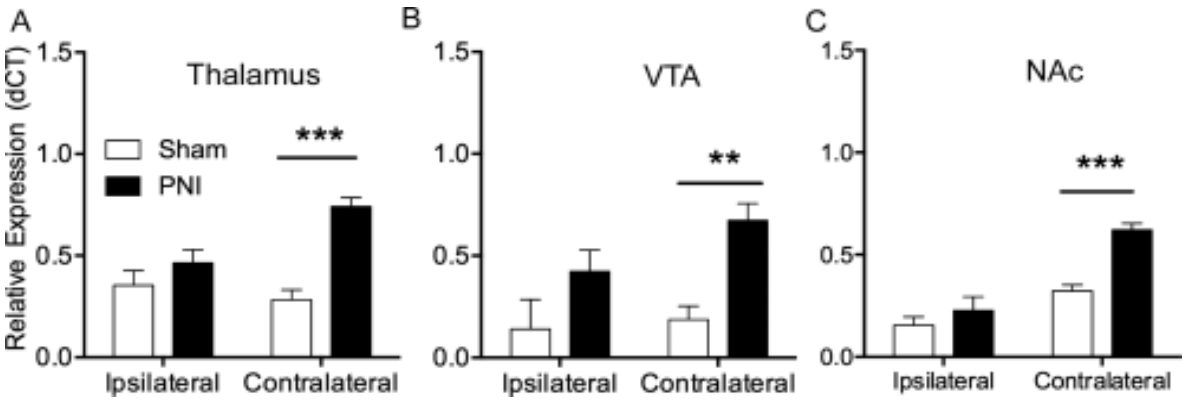

Figure 2.1. Relative Iba-1 gene expression in thalamus (A), VTA (B), and NAc (C) between sham versus PNI mice. Error bars are SEM, ** = P < 0.01, *** = P < 0.001.

After determining PNI-induced Iba-1 gene upregulation in multiple brain regions, we hypothesized that microglial activity would also be heightened in the presence of chronic pain. Thus, we conducted IBA-1 protein immunolabeling and found profound increases in many brain regions important for both sensory and affective pain transmission (Figure 2.2 and Table 2.2, n=6 per group). In most regions, microglial cell body size was significantly greater on the contralateral, but not ipsilateral, side of the PNI group. This was expected due to the decussation of pain signaling preferentially towards the contralateral side of the brain. In addition, we noted significant increase in cell body size within several somatotopic regions of the primary sensory cortex, including the region corresponding to the hindlimb ($F(1,10\ \text{side})=0.18$, $p=0.30$, $F(1,10\ \text{injury})=64.48$, ***$p<0.0001$, $F(1,10\ \text{interaction})=14.39$, **$p<0.01$) and head ($F(1,10\ \text{side})=6.26$, $p=0.09$, $F(1,10\ \text{injury})=0.58$, $p=0.46$, $F(1,10\ \text{interaction})=7.13$, *$p<0.05$). We also noted significant unilateral microglial activation in the habenula ($F(1,10\ \text{side})=13.72$, **$p<0.01$, $F(1,10\ \text{injury})=5.85$, *$p<0.05$, $F(1,10\ \text{interaction})=2.55$, $p=0.14$), and VTA ($F(1,10\ \text{side})=26.96$, **$p<0.01$, $F(1,10\ \text{injury})=3.98$, $p=0.07$, $F(1,10\ \text{interaction})=10.68$, **$p<0.008$). Interestingly, the NAc showed microglial activation on both the contralateral and ipsilateral side in the PNI group

(F(1,10 side)=0.29, p=0.59, F(1,10 injury)=10.25, **p<0.01, F(1,10 interaction)=0.33, p=0.57). The motor cortex did not show any change in microglial morphology on either side in the PNI group (F(1,10 side)=2.45, p=0.15, F(1,10 injury)=0.07, p=0.79, F(1,10 interaction)=0.56, p=0.47). No change in microglial cell body size was observed in any region on either the ipsilateral or contralateral side of the sham group. Total number of microglial cells did not differ between sham and PNI groups, nor between ipsilateral or contralateral sides in any of the brain regions measured (data not shown). Overall, the findings demonstrate that neuroinflammation can occur at regions that are not directly at the site of injury, highlighting the far-reaching ramifications of chronic pain and its complexity in the body.

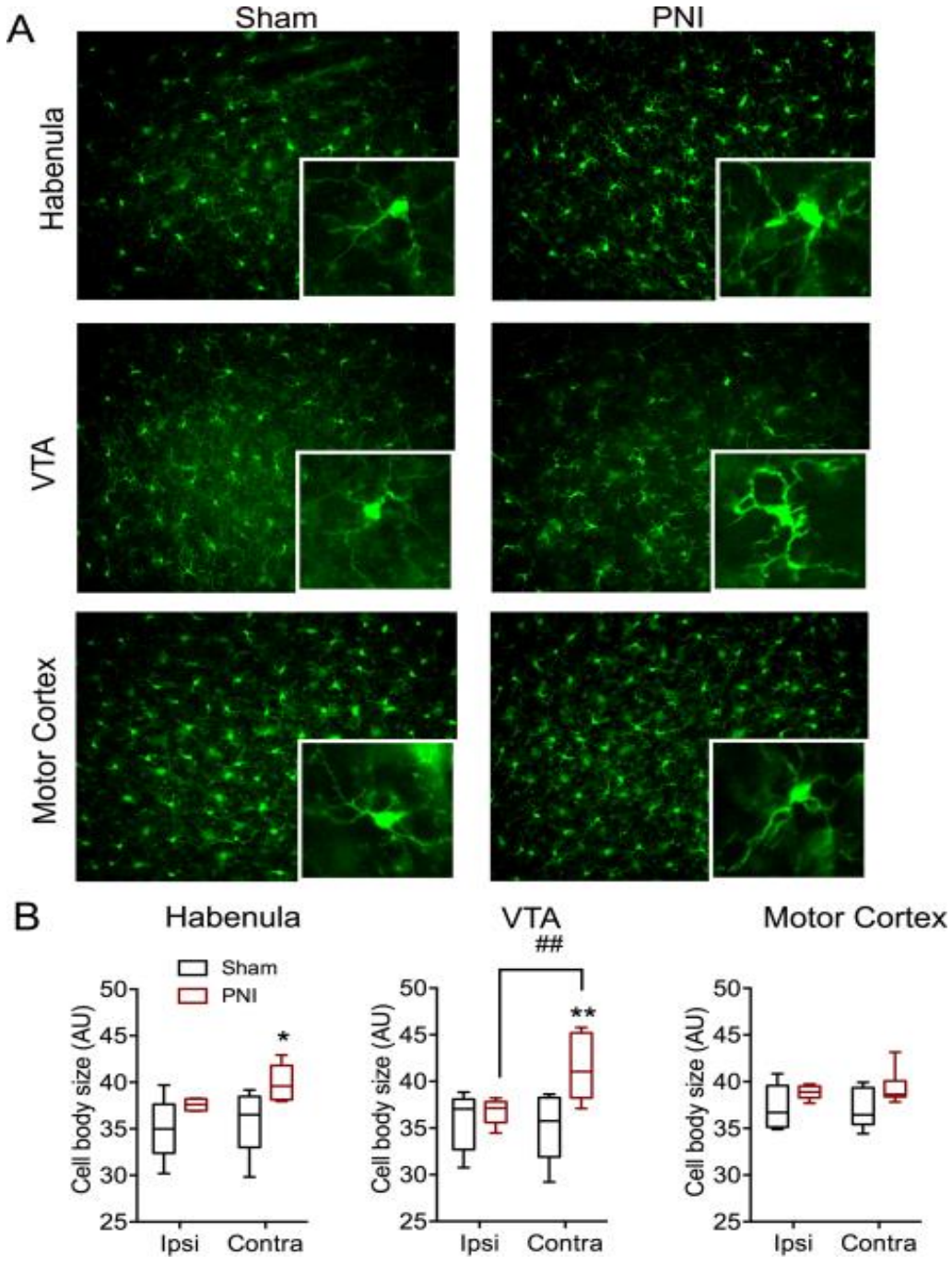

Figure 2.2. (A) Representative brain regions IHC-stained with IBA1 for microglia, comparing sham versus PNI. (B) Quantification of microglial soma between sham and PNI mice at the ipsi- versus contralateral hemispheres within habenula, VTA, and motor cortex. * = $P < 0.05$, ** = $P < 0.01$.

Brain Region	Sham		PNI	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
S1-J	36.7 (0.64)	38.0 (1.37)	36.25 (0.67)	38.84 (0.63) **, ϕ
S1-HL	35.5 (0.76)	34.1 (0.74)	39.7 (0.7)	42.3 (0.50) *, ϕϕ
MC-J	36.9 (0.75)	37.2 (0.79)	38.8 (0.59)	39.3 (0.59)
MC-HL	36.0 (0.44)	32.5 (0.77)	33.7 (0.64)	31.6 (1.01)
NAc	35.7 (0.67)	35.6 (0.37)	37.7 (0.72) #	38.5 (0.69) #
Amygdala	38.1 (0.3)	35.1 (0.80)	35.6 (0.52)	38.45 (1.56)
Habenula	35.0 (1.51)	35.9 (1.60)	37.6 (0.24)	39.9 (0.79) **, #
PAG	35.5 (2.14)	36.2 (1.34)	38.4 (0.45)	40.3 (0.83) *, #
VTA	35.7 (1.41)	35.2 (1.67)	36.8 (0.56)	41.4 (1.47) **, #, ϕϕ

Table 2.2. Microglial cell body size in isolated brain regions of control (sham) and peripheral nerve injury (PNI) groups. Data is expressed as average cell body size (in arbitrary units) with S.E.M. in parentheses. Abbreviations: S1-J, primary sensory cortex (Jaw); S1HL, primary sensory cortex (hind limb); MC-J, motor cortex (jaw); MC-HL, motor cortex (hind limb); NAc, nucleus accumbens; PAG, periaqueductal grey; VTA, ventral tegmental area. *=$p<0.05$, **=$p<0.01$ compared to ipsilateral side of same condition, #=$p<0.05$, compared to sham contralateral side, ϕ=$p<0.05$, ϕϕ=$p<0.01$ interaction between side and treatment.

Discussion

This study highlighted the impact of NP pain on brain microglial reactivity. Although it is understandable that neuroinflammatory mechanisms can occur at the injury site, as well as in the spinal cord at the level of primary afferent input, we have framed the magnitude of impact to be in many brain regions associated with sensory pain transmission as well as limbic brain structure, but it was not universal as some areas did not show increased reactivity such as the motor cortex. Based on these findings, the brain is highly affected by PNI and many neurochemical changes resulting from microglial activation could greatly shape how the brain functions during long-term pain. We focused especially on brain regions involved in the reward pathway because many mood-associated problems such as depression can arise in chronic pain, which may be due to microglial influence as evidenced in these results. Furthermore, it has been shown that microglial activation in the VTA impairs dopamine neuron activity and opioid reward via a BDNF-KCC2 pathway (Taylor et al., 2015).

It should be noted that an earlier study observed no microglial activation in higher cortical regions following a PNI (Zhang et al., 2008). The lack of microglial activation in this study could be due to the use of transgenically-labelled microglia (CX3CR1-GFP), given this line exhibits only one functional copy of the CX3CR1 receptor, impairments in pain-induced microglial activation might be expected (Jung et al., 2000). This would be particularly true in the brain, where the degree of microglial activation is not as robust as is observed in the ipsilateral spinal cord.

Previously, it was shown that chemotherapy-induced pain resulted in significant microglial activation throughout many brain regions including the PAG, thalamus, anterior cingulate cortex, secondary sensory cortex and medial forebrain bundle (Di Cesare Mannelli et

al., 2013). The current study extends these results by showing that even pain of a peripherally restricted origin is capable of activating microglia in diverse regions of the central nervous system. Notably, a recent study examining chronic pain patients has reported evidence for microglial activation in several of the same brain regions described in our current study, including the thalamus and sensory cortical regions (Loggia et al., 2015). Taken together, these studies form an emerging perspective recognizing brain microglia is positively correlated with the progression of chronic pain phenotypes.

The wide range of brain regions in which microglial activation was detected raises the question as to what signals or mechanisms are responsible for pain-induced microglial activation at sites far removed from the site of injury. Of note is the observation that the peripheral nerve injury did not induce microglial activation in all brain regions, such as the motor cortex.

The breadth of microglial activation throughout the brain suggests a potential for microglia to influence pain progression in a variety of brain regions. However, the research delineating the mechanisms by which microglia influence pain behavior in a region-specific manner is in its infancy. In the amygdala, CCR2 and IL1-β were implicated in the development of pain-induced anxiety (Sawada et al., 2014). This is particularly interesting because it alludes to the notion that neuroinflammation is a key contributor to the negative affect experienced in chronic pain. Given the robust activation of microglia that we observed in several brain regions, further research is warranted to identify novel areas in which microglia contribute to the development and progression of chronic pain. Although we have some ideas about how microglial signaling works in NP pain, these glial cells can release a host of other signaling molecules such as interleukin cytokines and other factors that can produce downstream changes in gene transcription throughout the brain. Such changes could have the potential to be

maladaptive and further studies may involve investigating other forms of neuroinflammation that occur after pain in an effort to link the multiple negative symptoms of pain together.

CHAPTER 3: REPEATED MILD TRAUMATIC BRAIN INJURY LEADS TO NEUROINFLAMMATION AND ALTERS BRAIN PLASTICITY

Introduction

Approximately 2.8 million people report sustaining a traumatic brain injury (TBI) annually (Taylor, CA et al., 2017). However, this number underestimates the true number of TBI cases each year, as many mild brain injuries go unreported. According to the Centers for Disease Control in the United States, 75% of reported cases of TBI are mild TBI, categorized as concussion-level injuries. Falls, intentional self-harm, motor vehicle accidents, military training, and sports-related incidents are the most common causes of this injury. TBI can have debilitating effects on patients, as it is unlike most other types of physical injury to the body. An injury to the body such as a broken limb is primarily a functional damage, which may recover given enough time. However, brain injuries do not heal in this manner. Furthermore, every brain injury has unique consequences that affect one's mood and behavior. Patients with brain injury can have symptoms immediately following the injury, or have delayed symptoms several weeks later. Unlike most other physical injuries, the mechanisms of the body trying to recover from TBI are largely unknown, which limit the effectiveness of current medical treatments.

TBI symptoms include epilepsy (Chen et al., 2017; Zimmermann, Martin, and Girgis, 2017), chronic NP pain (Irvine and Clark, 2017), dizziness (Akin et al., 2017), depression (Laliberte, Pereverseff, and Yeates, 2017; Yrondi et al., 2017), reduced cognition and social interaction (Howe et al., 2017). Depending upon context of the severity of the injury and its impact site, it is also highly co-morbid with anxiety disorders such as post-traumatic stress

disorder (Kaplan et al., 2017). In addition, persistent chronic headache is also one of the most commonly reported neurological disorders attributed to TBI, which severely negatively impact's patient quality of life (Stacey et al., 2017; Ellis et al., 2017; Suri et al., 2017; Mustafa et al., 2017). These symptoms taken together also leads to an increase risk of suicide among patients (Elman et al., 2013; Ilgen et al., 2013).

BDNF signaling has also been associated with pain hypersensitivities. BDNF is commonly released in various neuronal cell types, including sensory neurons (Acheson et al., 1995) and are highly involved in synaptic plasticity (Horch et al., 1999; Yamada and Nabeshima, 2003). This factor is highly implicated in pain processing. In particular, intrathecal injection of BDNF into the lumbar spinal area of rodents has been shown to reduce mechanical and thermal nociceptive thresholds (Li et al., 2008; Liang et al., 2014). While BDNF is known to be released in neurons, microglia can also release this factor (Batchelor et al., 1999). Prior studies have shown that TBI can induce microglial activation in the brain, which results in a release of BDNF and substance P (Nagamoto-Combs et al., 2007; Elliott et al., 2013). This notion ties into other published evidence that microglial reactivity via BDNF release can alter resting potentials in neurons by impairing chloride homeostasis via downregulation of potassium co-transporter KCC2 (Coull et al., 2005; Ferrini et al., 2013; Ferrini and De Koninck, 2013). This dysfunction of action potentials has been shown to negatively impact dopamine signaling in the reward pathway (Taylor et al., 2015; Taylor et al., 2016) as well as alter the function of brain regions that process mood and emotion (Taylor et al., 2017). Thus, this chapter discusses how TBI produces an increase in neuroinflammation within the mesolimbic brain system. Specifically, we show that TBI leads to pain hypersensitivity and negative affective-like behaviors, which are due to the upregulation of microglial activity in the brain.

Methods

Repeated Mild Traumatic Brain Injury

Mice were anesthetized using isoflurane (2.5%), heads shaved, and then transferred to the impactor foam bed platform. Sham animals were returned to home cage after this step. For TBI-assigned animals, continuous anesthesia was administered (1.5% isoflurane in oxygen) throughout the remaining procedures thereafter. The controlled cortical impact device parameters were set to 50 ms dwell, 1 mm depth, and 5 m/s speed. After the impact, mice were transferred to recovery station for up to 3 minutes to observe return of consciousness. Following the injury, animals were returned to home-cage and given liquid Tylenol diluted in water (3.2 ml in 250 ml water) ad libitum. For the repeated (5-hit) TBI group, this procedure was conducted over five consecutive days, once per day.

Minocycline Treatment of Animals

Minocycline hydrochloride (Sigma, M9511) was orally administered in drinking water (0.533 mg/ml) rather than systemic injections as this method of delivery is less stressful than twice daily i.p. injections. This protocol was shown to be effective in recovering spatial learning and hippocampal neurogenesis in aged mice (Kohman et al., 2013). Minocycline was administered (using black bottles to protect the drug from light) beginning in 5-weeks post-TBI and continued through the end of the study. The drug was replaced every 48h in fresh drinking water.

Forced Swim Test

To evaluate depressive-like behavior, we used the forced swim test that measures the total time mice spend immobile when forced to swim in an inescapable water tank (Porsolt, Le Pichon, & Jalfre, 1977). A mouse is considered to be immobile once swimming and escape behaviors have ceased, and it floats belly-down with its head above the water. The apparatus for this test was a cylindrical, acrylic water tank filled with water to 15 cm in depth. The apparatus resided on a table in a dark, quiet room. Water temperature was maintained at ~30 °C. The test session lasted six minutes, and the last four minutes of the test were analyzed for immobility time. Following the test session, the mouse was removed from the tank, dried with a towel, and placed in its cage on a heating pad for 15 minutes. Water in the tank was replaced with clean, 28 °C water following each trial. An experimenter monitored all test sessions for signs of drowning, which would result in immediate termination of the session, and transfer of the mouse to the heated cage. No signs of drowning occurred in any test session. Behavior was video recorded and two experimenters independently analyzed immobility blind to experimental condition.

Open-Field Test

Motor activity was measured in transparent acrylic cubic boxes (27.94 cm x 27.94 cm x 27.94 cm) with black-papered walls, each covered by a removable acrylic lid. Each mouse was placed in the center of a box and allowed to move freely for 5 minutes. Mouse activity was measured by a CCD camera linked to a computer running Ethovision behavioral tracking software (Noldus, Netherlands), capturing total distance traveled, time spent in an inside zone (square parameters set equidistant to all edges of the box with total area of 25% of the total arena

area) and an outside zone (the remaining 75% of the area of the arena), and mean velocity (cm/s).

Elevated Plus Maze (EPM)

This test involves an acrylic maze constructed in-house with two closed arms (50 x 10 cm), enclosed on three sides by 40 cm high walls, and two open arms (50 x 10 cm) with no walls, that intersect to form a "+" shape. The maze resides on a platform so that it rests 50 cm off the floor in a dark, quiet room. Mice were placed in the center of the maze facing toward one of the closed arms and were observed as they explored the maze for a period of 5 minutes. The number of open and closed arm entries were defined as three of the animal's paws crossing the border of an arm. The test also measured the total time each animal spent in the open and closed arms. In this well-validated paradigm, anxious mice spend less time in the open arms of the maze, and thus the objective measure of anxiety-like behavior is decreased time spent in the open arms of the maze, or alternatively, decreased entries into the open arms of the maze (Pellow et al., 1985). This can be calculated as follows:

Proportion of time spent in open arms =

[time in open arms (sec)] x 100 / total time in maze (sec)

Proportion of entries into open arms =

[# entries into open arms] x 100 / total entries into open or closed arms

The maze was cleaned with disinfectant following each test, and males and females were not tested on the same day to reduce exposure of mice to odors left behind by the opposite sex. Mouse activity was measured by a CCD camera linked to a computer running Ethovision behavioral tracking software (Noldus, Netherlands), which captured time spent in, frequency of entering, and latency to first entering open and closed arms.

Cold Plate Thermal Hyperalgesia Test

Cold sensitivity was assessed using the cold plate test (Bioseb, Vitrolles, France). Mice were placed on a metal plate maintained at 4 °C, within a large plexiglass cylinder. Before placing mice on the plate, 4 °C distilled water was sprayed onto the plate to increase thermal conductivity. Latency to the first jump was taken as an index of the cold nociception threshold, as was climbing the walls of the beaker with at least three limbs (one hindpaw). The cutoff was set at 30 seconds to avoid tissue damage.

Von Frey Tactile Allodynia Test

The Von Frey test measures mechanical thresholds in grams force of the plantar surface of the hind limbs of mice. Using filaments of differing force levels applied to the left hind paw, mice that experience pain sensitivity would retract their paws. Using the well-established SUDO method (Bonin, Bories, and De Koninck, 2014) derived from the original Up-Down method (Chaplan et al., 1996), paw withdrawal thresholds (in grams of force) to elicit a response 50% of the time are quantified for each mouse and compared between surgery groups. A reduced paw withdrawal threshold value suggests increased tactile allodynia. These tactile allodynia tests were conducted one week prior to TBI for a baseline measurement, and at multiple time points post-

TBI. Subsequent weekly Von Frey tests were conducted to determine whether tactile allodynia manifested in sham, 1-hit, or 5-hit TBI groups. Statistical significance was calculated using two-way ANOVA with Bonferroni multiple comparisons across each surgery group and sex.

Locomotor Test

Measurements for this test were obtained from distance traveled and velocity data recorded from EPM and open-field tests.

Nestlet Test

Effective nesting is an adaptive behavior that promotes self-preservation in mice and serves as a model for research on functional impairment of adaptive behavior by pain (Negus et al., 2015). At the start of each test session, mice were transferred from home cages to single-housed cages in a quiet, dark room, with a new 5 cm Nestlet bedding square (Ancare, Bellmore, NY). The Nestlet had been cut into 6 roughly equal pieces (1.7 cm x 1.7 cm), which were placed in the cage as shown in Figure 3.1. The mouse was allowed to move freely for 120 minutes and was then returned to its home cage. A blinded observer captured an image of each cage and manually scored the number of zones cleared (0-5) and the quality of the nest (0-4), based on position of Nestlet pieces and amount of shredding.

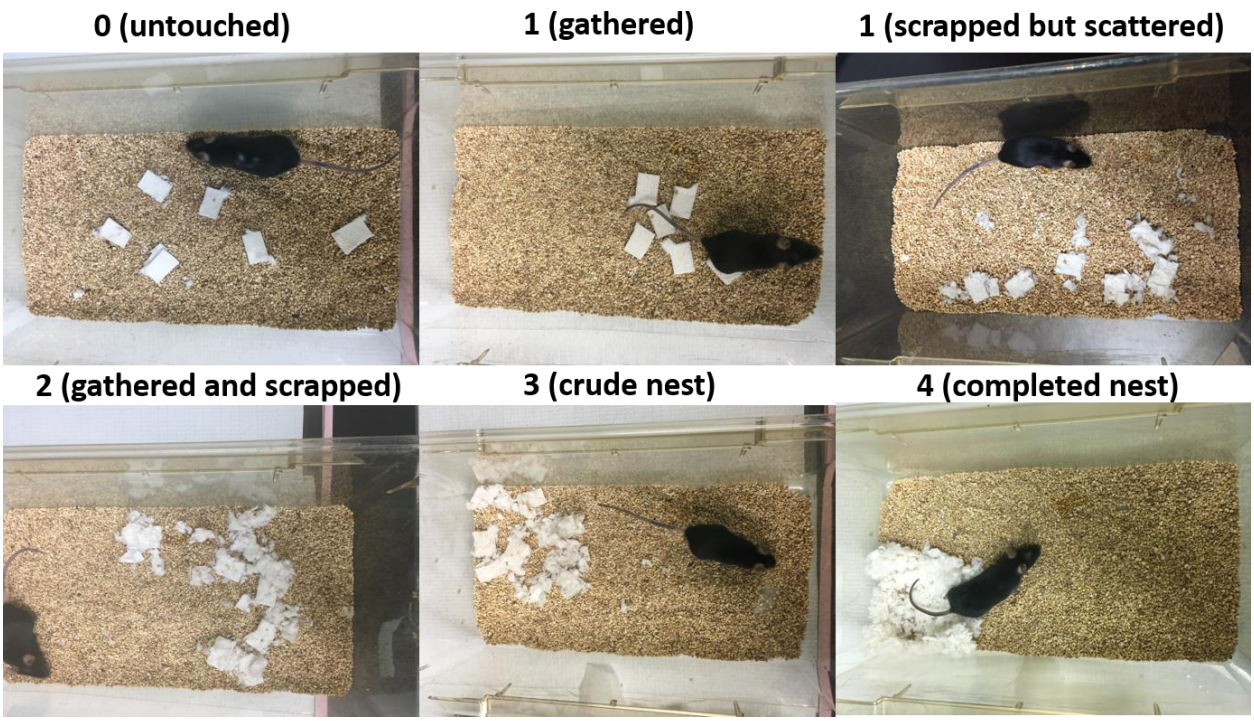

Figure 3.1: Nestlet test scoring paradigm.

RNAScope In Situ Hybridization

Brains were collected from sham versus 5-hit TBI, male versus female, vehicle versus minocycline-treated mice 8-weeks post-TBI. This corresponded to a total of 8 distinct treatment groups with three animals in each group. Brains were perfused with 4% paraformaldehyde in saline and snap-frozen with isopentane at -30 °C, and stored in -80 °C until further processing. Brains were coronal-sectioned via cryostat (18 µm thick) at -20 °C, and thaw-mounted on SuperFrost charged slides. Custom fluorescent probe labels were designed to fit complementary sequences on mRNA strands for BDNF gene (*Bdnf*) carrying fluorescent AlexaFluor488, tyrosine hydroxylase (*TH*) carrying Atto555, and IBA-1 (*Itgam*) carrying Atto647. Brain slices were incubated with 4% paraformaldehyde at 4 °C for 15 minutes, and then dehydrated with

50%, 70%, and 100% ethanol washes for 5 min each. Slides were then incubated overnight at -20 °C in 100% ethanol. Slides were then taken out and dried for 5 min and a hydrophobic barrier was drawn around the brain slices. Probes were activated in oven at 40 °C for 10 min. Slides were then incubated with probes following the RNAScope Multiplex processing kit (ACDBiosciences, Newark, CA). Slides were then cover-slipped and sealed with nail polish and stored in dark at -20 °C until visualization.

Quantitative RT-PCR

Brains were collected from 6 sham, 6 1-hit, and 6 5-hit TBI mice at 2-weeks post-surgery and were coronal-sectioned via cryostat (150 µm thick) at -20 °C and mounted on SuperFrost charged slides (Fisher Scientific, Pittsburg, PA). Tissue punches (1 mm diameter) were taken using a disposable biopsy plunger (Miltex, York, PA) for ACC, NAc, rostral hippocampus (HIPP), and VTA. Total RNA was collected from the brain tissue punches via Trizol extraction method (Ambion Life Technologies, Grand Island, NY). RNA was converted to cDNA using 100 U of M-MulV Reverse Transcriptase, 1 µM Oligo d(T)23VN, and 2 mM dNTP mix (New England Biolabs, Ipswich, MA), annealed at 70 °C for 10 min, extended at 40 °C for 1 hr, and inactivated at 95 °C for 5 min. Samples were chilled at 4 °C for 10 min and then stored at -20 °C. Real-time qPCR was conducted using primer sets for Kcc2, Bdnf, Itgam (IBA-1), and Cd11b control genes (Table 3.1). Using, 96-well optical plates (Applied Biosystems, Singapore), cDNA and PerfeCTa SYBR Green FastMix containing the primer sets (Quanta Biosciences, Gaithersburg, MD) were loaded and run on ABI ViiA7 fast block qPCR machine using cycling conditions in the PerfeCTa SYBR Green FastMix manual. Cycle threshold outputs were calculated and normalized to the actin housekeeping gene to compute ΔCT. Relative expression

levels were determined by normalizing sham and NP pain groups to 3 same-age naïve mice brain samples via ΔΔCT method. The results were statistically analyzed using one-way ANOVA with Bonferroni correction across surgery groups for each brain region.

Sequence	Primer
CGC ATG TTG TTC AGC ATG G	KCC2-R
GGC ACC ACC TTT GCT GG	KCC2-F
CAG CAT TCG CTT CAA GGA CAT A	IBA1-R
ATC AAC AAG CAA TTC CTC GAT GA	IBA1-F
CCG CCA GAC ATG TCC AC	BDNF-R
CAC TCC GAC CCT GCC CGC	BDNF-F
CCA GTT GGT AAC AAT GCC ATG T	Actin-R
GGC TGT ATT CCC CTC CAT CG	Actin-F
CAT CAT GTC CTT GTA CTG CCG CTT G	CD11b-R
CAG ATC AAC AAT GTG ACC GTA TGG G	CD11b-F

Table 3.1: List of primers used in qRT-PCR on sham versus TBI mice brain tissue.

Immunohistochemistry

Brains were collected from sham versus 5-hit TBI, male versus female, vehicle versus minocycline-treated mice 8-weeks post-TBI. This corresponded to a total of 8 distinct treatment groups with three animals in each group. Brains were perfused with 4% paraformaldehyde in saline and snap-frozen with isopentane at -30 °C, and stored in -80 °C until further processing. Brains were coronal-sectioned via cryostat (20 µm thick) at -20 °C, and stored in 96-well optical assay plates (1 slice per well) in PBS at 4 °C until ready to process. On day of processing, slices were transferred into 24-well culture plates and incubated in blocking buffer for 1 hour at RT using 3% Normal Goat Serum (NGS) + 5% bovine serum albumin (BSA) in 0.1M PBS at approximately 40 rpm shaking platform to allow free-floating antibody binding. Blocking buffer was then aspirated and primary antibody solution (0.5% NGS + 1% BSA in 0.1M PBS with antibody dilution factor described in Table 2) was added to the wells containing the brain slices.

The samples were incubated in 4 °C overnight with shaking at 40 rpm. Samples were then washed 3 times with 0.1M PBS at RT for 10 minutes per wash at 40 rpm. Secondary fluorescent antibody in 0.5% NGS + 1% BSA in 0.1M PBS (dilution factor in Table 2) was incubated at 40 rpm for 2 hours at RT in the dark. Brain slices were mounted to sterile glass microscope slides and glued onto cover slip using AntiFade Polymount containing DAPI (Polysciences, Inc., Warrington, PA). Slides were stored in the dark at RT to dry overnight. Slides were finally sealed with fast-dry nail polish and stored at 4 °C until ready to visualize via Nikon Ti-E inverted widefield fluorescence microscope. Image fluorescent signal were quantified using FIJI, an extension package software of ImageJ. Statistical analysis was conducted via 3-way ANOVA with Bonferroni multiple comparisons across sex, surgery, and drug treatment groups.

Western Immunoblotting

Brains were collected from 4 sham, 4 1-hit, and 4 5-hit TBI mice at 2-weeks post-surgery and snap-frozen with isopentane at -30 °C, and stored in -80 °C until ready to be sectioned. Brains were coronal-sectioned via cryostat (150 µm thick) at -20 °C, mounted on SuperFrost charged slides, and tissue punches (1 mm diameter) were taken using a disposable biopsy plunger for ACC, NAc, rostral HIPP, and VTA. Tissue punches were homogenized in lysis buffer (50 mM Tris-Base, 4 mM EDTA, pH 7.4) with protease inhibitor cocktail (Pierce Biotech - Thermo Scientific, Rockford, IL), centrifuged at 10000 x g to remove DNA/debris, and supernatant protein was extracted and stored at -20 °C. Protein concentrations were analyzed using Pierce^{TM} micro BCA assay kit (Thermo Fisher, Walham, MA). Protein samples were mixed with NuPAGE LDS Loading Buffer and reducing reagent (Novex, Carlsbad, CA), heated to 70 °C for 10 min, and stored at -20 °C to be used for gel electrophoresis. Gel electrophoresis

of protein samples was conducted using Invitrogen SDS-PAGE gel box, NuPAGE MES Running Buffer and Bis-Tris Mini Gels (Novex, Carlsbad, CA), and loaded alongside PAGE-Ruler Plus Prestained Protein Ladder (Fisher Scientific, Pittsburg, PA), run at 120 V for 2 hours. Proteins from gels were transferred onto nitrocellulose (0.45 µm pore size) (Novex, Carlsbad, CA) using BioRad protein transfer box at 4 °C and 250 mA for 50 min. Membranes were blocked with 5% milk and TBS-T (1%) at RT for 1 hour. Membranes were subsequently incubated with primary antibodies overnight at 4 °C. After primary antibody incubation, membranes were washed 3x with TBS-T (10 min per wash) and anti-rabbit or anti-goat antibodies were then incubated at 1:2000 dilution in 5% milk and TBS-T (1%) for 90 min at RT with gentle shaking. The membranes were washed again 3x with TBS-T (10 min per wash), followed by 1 min revelation of membranes using Amersham ECL-Plus substrate (GE Healthcare, Visalia, CA). The membranes were visualized with Li-Cor Odyssey Fc Imager. Membranes were then washed and antibody-stripped using glycine stripping buffer (200 mM glycine, pH 2.6) with shaking at RT for 1 hour, then washed 3x with TBS-T (10 min per wash). Membranes were re-probed with beta-actin antibody or GAPDH antibody overnight at 4 °C, and subsequent secondary antibody for 1 hour at RT. Band intensities were quantified using Li-Cor Image software and normalized to beta-actin or GAPDH control bands.

Primary Antibody	Catalog	Description	Dilution Factor
Anti-PSD95 [EP2652Y]	Abcam: ab76115	Rabbit monoclonal	1:200
Anti-tyrosine hydroxylase	Abcam: ab112	Rabbit polyclonal	1:500
Anti-tyrosine hydroxylase	Millipore: MAB318	Mouse monoclonal	1:500
Anti-KCC2 co-transporter	Millipore: 07-432	Rabbit polyclonal	1:200
Anti-BDNF [EPR1292]	Abcam: ab108319	Rabbit monoclonal	1:200
Anti-GAPDH [6C5]	Abcam: ab8245	Mouse monoclonal	1:2000
Anti-beta actin [AC-15]	Abcam: ab6276	Mouse monoclonal	1:5000
Secondary Antibody	Catalog	Dilution Factor	
Donkey anti-Mouse IgG, HRP	Thermo Fisher: A16017	1:5000	
Goat anti-Chicken IgY, HRP	Thermo Fisher: A16054	1:4000	
Goat anti-Rabbit IgG, HRP	Thermo Fisher: G-21234	1:4000	

Table 3.2: List of antibodies used for immunoblotting and immunohistochemistry.

Taqman Array Assay

Brains were collected from 1 sham, 1 1-hit, and 1 5-hit mouse at 2-weeks post-surgery, and were coronal-sectioned via cryostat (150 µm thick) at -20 °C and mounted on SuperFrost charged slides (Fisher Scientific, Pittsburg, PA). Tissue punches (1 mm diameter) were taken using a disposable biopsy plunger (Miltex, York, PA) for ACC, NAc, rostral HIPP, and VTA. Total RNA was collected from the brain tissue punches via Trizol extraction method (Ambion Life Technologies, Grand Island, NY). RNA was converted to cDNA using 100 U of M-MulV Reverse Transcriptase, 1 µM Oligo d(T)23VN, and 2 mM dNTP mix (New England Biolabs, Ipswich, MA), annealed at 70 °C for 10 min, extended at 40 °C for 1 hr, and inactivated at 95 °C for 5 min. Samples were chilled at 4 °C for 10 min and then stored at -20 °C. Samples are loaded into a pre-designed Taqman array (Applied Biosystems, Foster City, CA), sealed with a micro fluidic card sealer, and run with ABI Viia 7 real-time PCR machine, following cycling conditions specified in the Taqman array manual (Life Technologies, Carlsbad, CA). The results are recorded on Excel multi-tab spreadsheet and imported into Ingenuity Pathway Analysis (Qiagen, Valencia, CA) for network cluster analysis, quantification, and gene heatmap generation.

Corpus Callosum Analysis

Brain slices were collected via cryostat at -20 °C and sliced at 30 µm thickness. All sections were collected in sequence. The sections were stained with Cresyl violet and then analyzed using StereoInvestigator software v11.08.01 (MicroBrightField, Inc., Williston, VT) at 12-slice intervals. A 100 µm cross-sectional grid was laid down to assess corpus callosum volume and

coefficient of error for each animal was less than 0.10. Corpus callosum boundaries were set based on identifying the first and last sections that contained cross-hemisphere connections.

Statistics and Data Analysis

For pain threshold tests, withdrawal thresholds were averaged among groups and compared by 1-way repeated measures ANOVA with Bonferroni post-hoc tests, and significance set at $P < 0.05$. Male and female cohorts were analyzed separately for those experiments. For the negative affect behavior tests, statistical analysis was done using 2-way ANOVA between sham versus 5-hit and males versus females, with Bonferroni multiple corrections. Significance threshold was set at $P < 0.05$. For corpus callosum volume, a 2-way ANOVA was conducted between sham and 5-hit TBI and males versus females, with Bonferroni multiple comparisons and $P < 0.05$. For qRT-PCR and immunohistochemistry, 1-way ANOVA was conducted between sham and 5-hit TBI male animals, with Bonferroni multiple comparisons and $P < 0.05$. Correlation analyses between corpus callosum volume and negative affect was conducted with Pearson's Product-Moment Correlation tests to measure degree of linearity between the two variables. Statistical significance was set at $P < 0.05$. For the negative affect behavior tests that involved minocycline treatment, we initially examined the males versus females variable in a three-way ANOVA and did not find statistical significance by sex. Using Power Analysis tests, we determined that a larger sample size would be necessary to determine such significance. Instead, to increase overall power of the study, we combined both male and female groups. Further statistical analysis was done using 2-way ANOVA between sham versus 5-hit and vehicle versus minocycline treatment, with Bonferroni multiple corrections. Significance threshold was set at $P < 0.05$.

Results

Repeated TBI leads to thermal hyperalgesia but not tactile allodynia

In this study, it was observed that female, but not male, 5-hit TBI animals developed cold hyperalgesia (Figure 3.2A). A significant decrease in cold thermal thresholds occurred at 4-weeks post-injury in females and continued through 7-weeks post-injury. Although we saw thermal pain sensitivity in TBI mice, tactile allodynia was not present as von Frey thresholds were not different between rmTBI mice and sham controls (Figure 3.2B). There were also no sex differences in paw withdrawal threshold, suggesting that mechanical allodynia pathways have are regulated similarly in both sexes, and thermal hyperalgesia may have sex-dependent regulation in the TBI model.

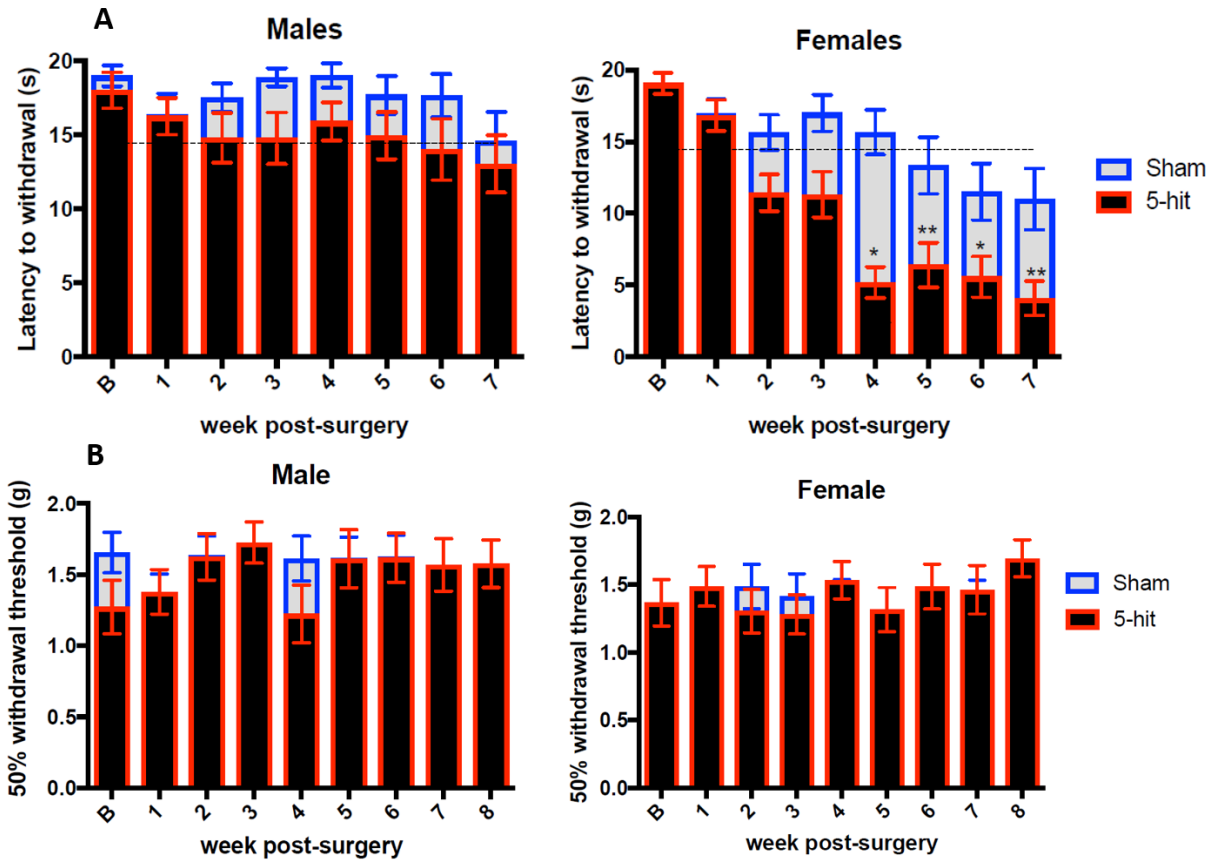

Figure 3.2: Pain threshold tests for **(A)** cold thermal hyperalgesia via cold plate test and **(B)** tactile allodynia via Von Frey test.

Anxiety and depressive-like behavioral observations following rmTBI

Both male and female 5-hit TBI animals showed an increase in time spent in the open arms of the EPM and an increase in the frequency of entering the open arms compared to sham counterparts (Figure 3.3A). Although these results may initially indicate decreased anxiety, the EPM test is also described to study risk assessment behavior (Rodgers et al., 1999; Macri et al., 2002; Albani et al., 2015). When we conducted the open-field test, we found that both male and female 5-hit TBI animals spent less time in the center zone of the open-field test (Figure 3.3B), which suggests that repeated TBI can lead to increased anxiety in this paradigm. Similarly, unexpected results were demonstrated in the 5-hit TBI mice in a model of depression where

rmTBI decreased immobility time in the forced swim test (Figure 3.3C). While we predicted that 5-hit TBI mice should develop anxiety and depressive-like behaviors, it is possible that other behavior patterns resulting from the injury were masking such psychopathologies. Thus, we also looked at their locomotor behavior across motion tracks from the EPM and open-field tests. It was observed that 5-hit TBI mice had increased locomotor activity (distance traveled and velocity during each of those tests compared to sham mice). Furthermore, this hyper-locomotor activity was seen in both males and female rmTBI mice (Figure 3.3D). This hyper-locomotor activity confounds both the forced swim test and the EPM test. Although we hypothesized that there were would be decreased nest-building behavior in the nestlet test, there were no significant differences between the sham and rm TBI groups in either the male or female animals (data not shown); however, we did notice that by collapsing all surgery groups and comparing males and females, the males overall had slightly higher nestlet test scores. These results taken together indicate that rmTBI animals may have some maladaptive behaviors which include anxiety and potentially greater risk-taking than sham animals. They also have noticeable hyperactivity that could be another psychopathology resulting from the injury. Overall, we were still able to demonstrate that rmTBI animals showed some forms of anxiety in the open-field test.

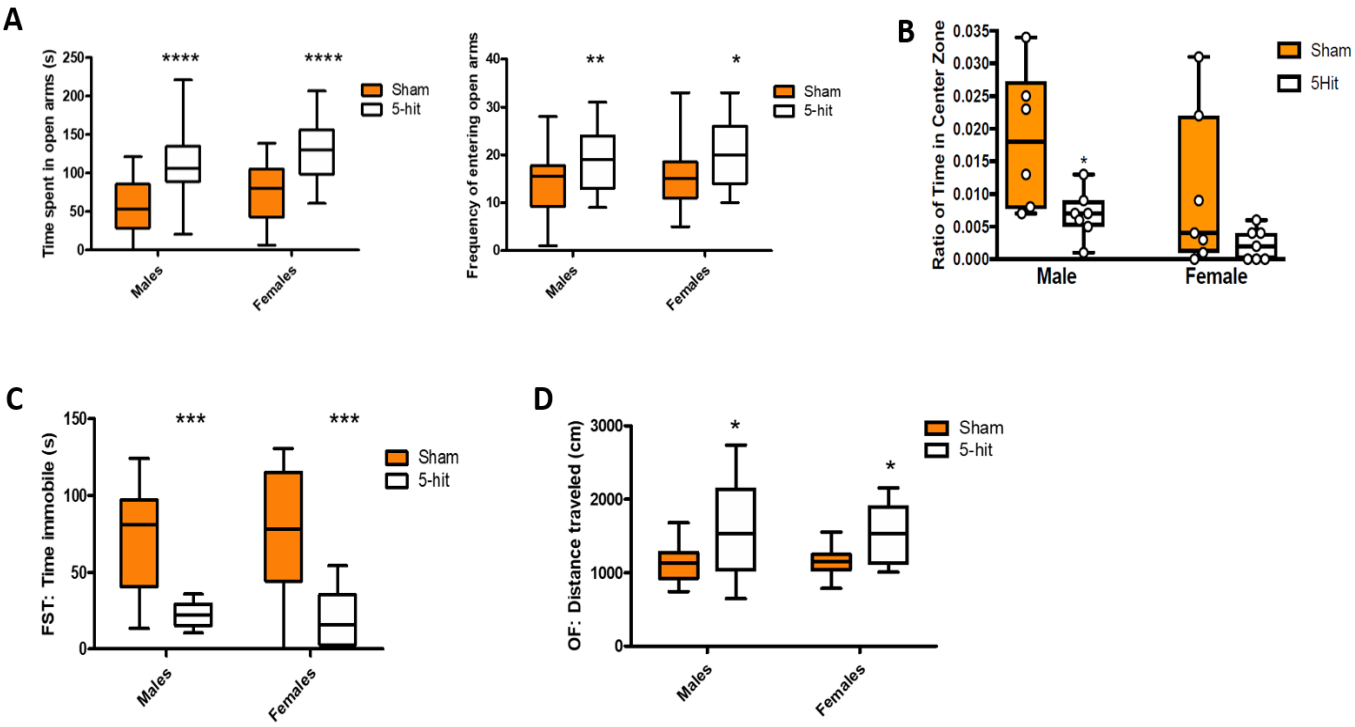

Figure 3.3: Behavior tests in sham versus 5-hit TBI mice. **(A)** Elevated plus maze for anxiety and risk-taking behavior. **(B)** Open-field test for anxiety. **(C)** Forced swim test for depressive-like behavior. **(D)** Locomotor test for hyperactivity. Statistical analysis were done using 2-way ANOVA between sham versus 5-hit and males versus females, with Bonferroni multiple corrections. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05

Neuroanatomical changes and inflammation following TBI

The corpus callosum is important in maintaining communication across hemispheres in the brain. Damage to this region is also associated with mood disorders (Bhatia, Saha and Doval, 2016; Emsell et al., 2017; Dretsch et al., 2017; Gifuni et al., 2017; Van Schependom et al., 2017; Kim et al., 2017). White matter abnormalities have been studied in humans following multiple concussions or traumatic brain injuries, which led to neuropsychiatric symptoms and cognitive deficits (Johnson et al., 2013; Multani et al., 2016). Because of this, we collected the corpus callosum (CC) from sham and 5-hit TBI mice. Corpus callosum volume was measured and determined to be decreased in both male and female rmTBI groups compared to their sham counterparts (Figure 3.4A). Next, we postulated that this decrease in volume was due to neuroinflammation in the brain after injury. The marker Iba1 was used to label microglial cells throughout the brain, which are known to be major contributors to neuroinflammation. Immunohistochemistry studies in these animals showed that Iba1 was significantly increased in rmTBI mice brains compared to sham (Figure 3.4B). This was seen in both males and females. BDNF gene expression was upregulated in rmTBI animals compared to sham counterparts (Figure 3.4C). Because this factor is secreted by microglia and neurons, it is evident that brain injury leads to their heightened activity. Furthermore, the pro-inflammatory gene Cd11b expression was upregulated at the ACC, NAc, and VTA these mice (Figure 3.4D), with no significant change at the hippocampus. Cd11b is expressed in leukocytes throughout the body as well as glia in the nervous system (Solovjov, Pluskota, and Plow, 2005), and mediates their migration and adhesion to tissue areas where they can trigger immune response. The upregulation of Cd11b in observed in reward pathways following rmTBI implies the likelihood of mood and behavioral alterations in these animals. In addition, since these data reflect gene

expression, it is likely that these changes in rmTBI would involve a *prolonged* mechanism of action that leads to sustained neuroinflammation after such injury, which would therefore result in long-term negative phenotypes. rmTBI mice exhibited significantly decreased levels of PSD95 in our immunohistochemistry experiment in the ACC and hippocampus (Figure 3.4E). Similar results were observed in our immunoblotting experiments, though the band quantification data did not reach statistical significance (data not shown). PSD95 is a synaptic protein found at terminals of neuronal junctions. The loss of PSD95 after brain injury implies a loss of synapses and the likelihood of either neuronal death or reduction in the strength of neuronal signaling in these brain regions. Because we observed this decline in PSD95 in the ACC, a region responsible for attention, motivation, and emotion (Bush, Luu, and Posner, 2000; Allman et al., 2001), we expected that TBI animals may have cognitive deficits and negative affect. Furthermore, the hippocampus is highly important for learning and memory storage and recall (Van Elzakker et al., 2008; Eichenbaum and Cohen, 2014). The decrease in PSD95 in this region suggests a remodeling of the hippocampal neurons that could negatively impact the formation of memory.

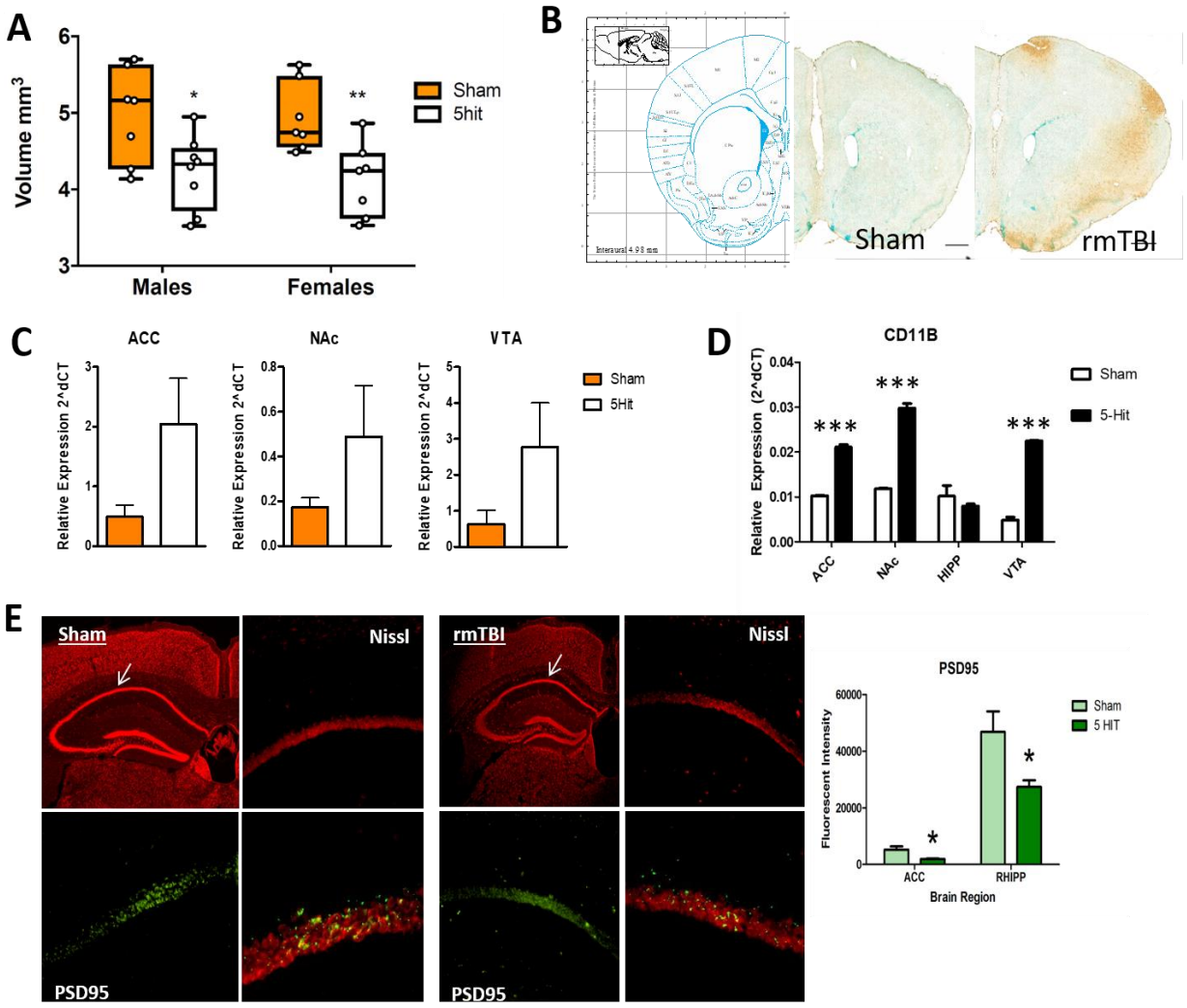

Figure 3.4: Neuroanatomical changes and inflammation after TBI. **(A)** Corpus callosum volume in mm^3. Statistical analysis was done using two-way ANOVA between sham versus 5-hit TBI and males versus females, with Bonferroni multiple comparisons. **P<0.01, *P<0.05 **(B)** Representative images of DAB staining for microglial marker, IBA-1 in striatal sections containing prefrontal cortex, caudate, and nucleus accumbens. **(C)** BDNF relative gene expression was slightly upregulated in 5-hit compared to sham mice. Student's t-test for each brain region comparing sham versus 5-hit showed no significance in these regions, although a trend towards significance existed (P<0.15 for all regions). **(D)** Proinflammatory marker CD11B relative gene expression via qRT-PCR in ACC, NAc, HIPP, and VTA brain punches of male animals. Statistical analysis was done using one-way ANOVA between sham versus 5-hit TBI within each brain region with Bonferroni corrections. ***P<0.001. **(E)** Immunofluorescence of PSD95 synaptic marker in sham versus 5-hit rmTBI mice. PSD95 protein was quantified within the ACC and HIPP, and was found to be significantly reduced in 5-hit compared to sham animals. One-way ANOVA with Bonferroni post-hoc was conducted comparing surgery groups within each brain region. *P<0.05

To connect the physiological effects of TBI on the brain to the behavioral outcomes in these animals, correlation analyses were conducted to match individual animal data points together. From this study, there was a significant correlation of decreased CC volume to the increased cold hyperalgesia experienced by the male, but not female mice (Figure 3.5A). This decrease in CC volume was not significantly correlated with the hyperlocomotion in these mice, regardless of sex (Figure 3.5B). There were however, sex differences in correlation analyses between CC volume and risk-taking, and between CC volume and anxiety in the open-field test. Female 5-hit TBI animals with smaller CC volume were associated with greater risk-taking behaviors (Figure 3.5C), whereas male 5-hit TBI animals with smaller CC volume were associated with greater anxiety (Figure 3.5D). Based on these results, there are notable sex differences in terms of the importance of corpus callosum anatomy to rodent behavior. Changes to the corpus callosum may impact pain thresholds more significantly in males than females. In terms of risk-taking, the corpus callosum may play a more important role in females than in males.

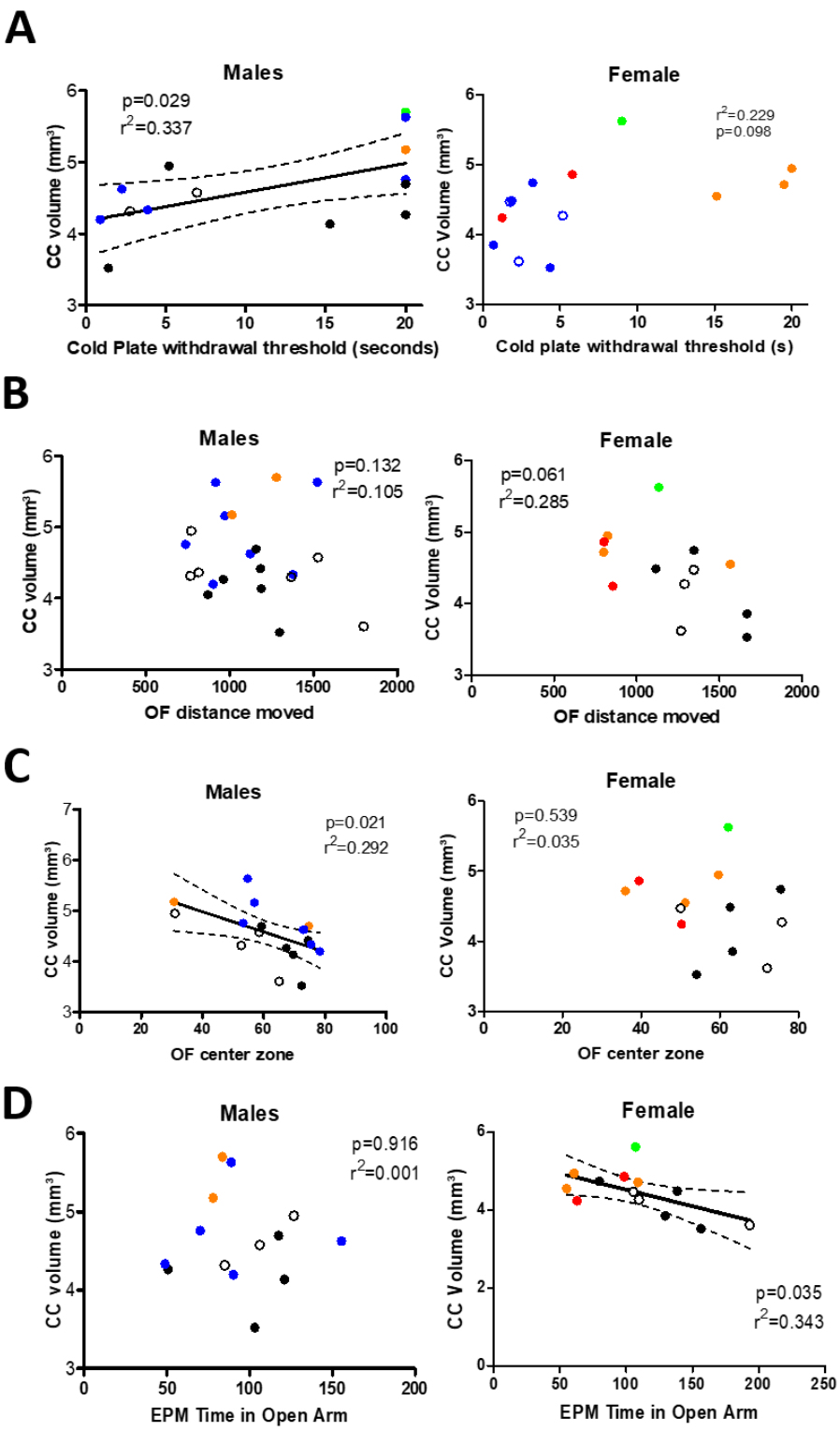

Figure 3.5: Correlation analysis between corpus callosum and behavior (collapsed across all surgery groups) in male and female mice. Statistical analyses were done using Pearson's Product-Moment Correlation tests to measure degree of linearity between the two variables. $P<0.05$ is defined as statistically significant. **(A)** Corpus callosum versus cold plate withdrawal hyperalgesia. Males show significant positive correlation whereas females show no correlation. **(B)** Corpus callosum versus total distance traveled in open-field arena. Neither males nor females showed correlation, although there is a trend towards negative correlation in females ($P=0.061$). **(C)** Corpus callosum versus time in center zone in open-field arena. Males showed significant negative correlation whereas females show no correlation. **(D)** Corpus callosum versus time in open arm of the EPM test. Males showed no correlation whereas females showed significant negative correlation.

Microglial activation contributes to TBI psychopathology

After establishing the link between neuroinflammation and the changes in pain, anxiety, and risk-taking behaviors in TBI mice, we next assessed whether these phenomena are specifically due to microglial activation following the brain injury. Prior studies have indicated that microglial activation in chronic pain contributes to the pain aversion as well as a disruption in mesolimbic dopamine signaling (Taylor et al., 2015; Taylor et al., 2016). Based on the results in Figure 3.4, it was likely that microglia in 5-hit TBI animals also contribute to the behaviors described in Figure 3.3. Therefore, we administered minocycline, a microglial inhibitor, in the water of sham versus rmTBI mice beginning at week 4 post-injury and continued until the end of the study. The same behavior test studies were conducted on these minocycline-treated mice beginning 1 week following treatment and compared to their vehicle-treated counterparts. It was observed that minocycline-treated rmTBI male mice displayed significantly less cold hyperalgesia than vehicle-treated rmTBI male mice (Figure 3.6A). This effect was not seen in females, which suggests that minocycline's effect to inhibit microglial involvement in thermal pain processing was a sex-specific effect. We initially studied the sex-dependent effects of minocycline on the negative affect behaviors of sham versus rmTBI mice. However, due to measuring the factor of inhibiting microglia with minocycline, we did not have a sizeable sample to see significant differences in the behavioral changes between male and female animals. Statistical analyses between male and female groups showed no difference, so to increase the power of the study, we combined the sample sizes of both groups and compared the effects of minocycline in sham and rmTBI animals. We observed in the combined male and female group, minocycline-treated rmTBI mice displayed reduced anxiety in the open-field test compared to the vehicle-treated rmTBI mice (Figure 3.6B). However, minocycline had no effect in reversing

risk-taking behavior in the elevated plus maze (Figure 3.6C), depressive-like behaviors in the forced swim test (Figure 3.6D), or hyperlocomotion in an open-field arena (Figure 3.6E) suggesting dynamic microglial signaling pathways and possible other mechanisms are involved that lead to the multitude of maladaptive behaviors observed.

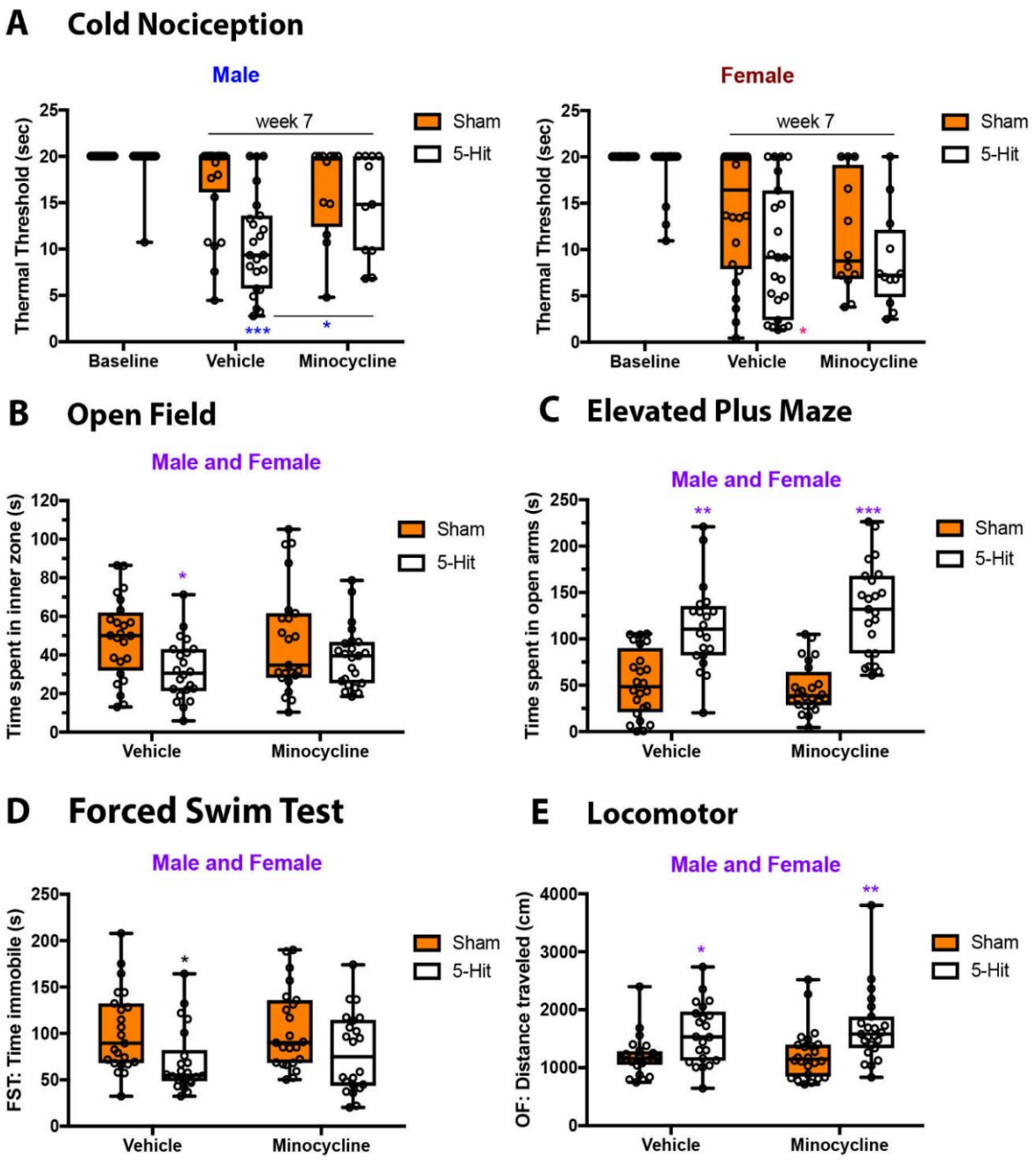

Figure 3.6: Effect of microglia activity on pain hypersensitivity and behavior in sham versus 5-hit TBI mice. Animals were given either vehicle or microglia inhibitor minocycline orally during the 7th week after sham or 5-hit TBI procedure. After treatment, behavior tests were conducted. Sex differences were noted in the cold nociception test; however, there were no sex differences in the other behavior tests, and thus these data were collapsed. **(A)** Cold nociception thresholds. **(B)** Open-field test. **(C)** Elevated plus maze. **(D)** Forced swim test. **(E)** Locomotor test. Statistical analyses for all tests were done using two-way ANOVA between sham versus 5-hit TBI and vehicle versus minocycline with Bonferroni corrections. *P<0.05, **P<0.01, ***P<0.001

Discussion

This novel study links behavioral effects and microglial activity in the brains of rmTBI rodents. We showed that rmTBI leads to changes in rodent thermal but not tactile pain sensitivity, suggesting that rmTBI alters pain pathways differentially. The increase in thermal pain hypersensitivity was also found to be a sexually dimorphic effect, suggesting different neuroplasticity mechanisms shaping pain perception between males and females. Our observations indicate that TBI may lead to chronic (possibly NP) pain, and further adds to the literature that these two phenomena are greatly associated with one another.

We identified decrease in brain corpus callosum volume after rmTBI, which was expected considering the impact site was near this region of the brain. More important, the corpus callosum volume decline was correlated to thermal pain hypersensitivity as well as anxiety-like behavior, which alludes to the importance of this brain region in terms of TBI psychopathology. Changes in brain volume have also been associated with other secondary illnesses such as chronic headache (Schwedt et al., 2017). Migraines have been correlated with brain injury in past studies (Beswick-Escanlar et al., 2016; Lucas and Blume, 2017). Furthermore, migraines may also lead to pain sensitization (Lou et al., 2017; Wilbrink et al., 2017). Although our study did not probe for headache-like symptoms in our rodent model of rmTBI, it is possible that these animals may also develop such symptoms following the procedure, which may correlate to the changes in pain sensitivity after brain injury in this manner.

We observed significant levels of inflammation in the brains of rmTBI mice and ascertained the microglial influence throughout the reward circuitry following injury. In particular, we noted that microglial marker Iba-1 was found in the NAc. The increase in Iba-1

corresponded to a slight increase in BDNF mRNA expression in the reward pathway (although not statistically significant). Furthermore, proinflammatory CD11B mRNA (also tied to microglial activity) was highly upregulated in the ACC, NAc, and VTA. The overall increase in neuroinflammation alluded to the idea that these glia cells were influencing the behavior of TBI animals. One of the major known functions of microglia in the brain is their ability to prune synapses and facilitate neuroplasticity by making room for new synaptic development (Miyamoto et al., 2016). However, their dysregulation or over-activation can lead to negative consequences in synaptic formation (Paolicelli and Ferretti, 2017). The BDNF-TrkB pathway has been shown to modulate PSD95 expression and neuronal apoptosis (Wu, Wu, and Guo, 2017) as well as regulate hippocampal dendritic mRNA metabolism and synaptic NMDA receptor expression (Leal et al., 2017). This idea tied together the role of microglial activity to brain remodeling following TBI. After observing the dramatic increase in microglial activity in the reward pathway in TBI mice, we predicted that they would reduce synapses in various regions of the brain. Indeed, we found that PSD95 synaptic marker was significantly reduced in the ACC and hippocampus, regions that are important for cognition and mood processing. Overall, the increase in proinflammatory markers and the subsequent decrease in synapses (and changes in brain plasticity) may be one of the many mechanisms that explains how TBI causes negative behavioral consequences.

Based on the findings from Chapter 2, it is possible that subsequent mood-related behavioral decline caused by microglia in that particular chronic pain scenario would also be present in our rmTBI model since we observed similar microglial activity in the same brain regions. We hypothesized in this study that the negative affect behaviors following brain injury could be reversed by inhibiting the upregulation of microglial activity. Using minocycline to

block microglia, we indeed observed a decrease in anxiety behavior in the open-field test. However, microglial activity was not the driver of risk-taking nor hyperactivity of these animals as minocycline did not decrease open-arm time in the EPM and did not reduce total distance traveled in the locomotor test, respectively. These findings show selective roles for microglia in the brain after TBI and suggest that neuroinflammation stemming from the rise in microglial activity is only one factor in a diverse pool of factors that all contribute to the negative consequences of TBI.

In summary, the study outlined in this chapter provides insight to understanding the mechanisms of neuroinflammation and behavioral outcomes following brain injury. The mechanistic links uncovered in Chapters 2 and 3 together open novel possibilities whereby developing treatments for mood disorders during chronic pain can also be implemented in TBI victims as well. Furthermore, our study pinpoints the therapeutic target for such treatments to be modulating microglia activity specifically in the brain.

CHAPTER 4: THE ROLE OF KAPPA OPIOID RECEPTOR IN CHRONIC PAIN PSYCHOPATHOLOGY

Introduction

The KOR is expressed in many regions in the brain, and is crucial to regulating many neural signaling networks, such as the reward pathway, mood, and pain (Bruijnzeel, 2009; Sun, 2011; Lalanne, et al., 2014). Because KOR activation can lead to attenuation of nociception as well as place aversion to KOR agonists, there have been efforts to elucidate the receptor distribution among different regions in the brain. Some of the oldest studies involved administering KOR agonists into mice while measuring glucose consumption in different regions of the rodent brain (Ableitner & Herz, 1989). More modern techniques to measure KOR distribution and activation hotspots have been done using KOR-Cre knock-in of the receptor and trace its localization (Cai, et al., 2015). These studies provide cursory maps on where KOR is highly involved to regulate the different signaling networks. Other attempts have been made to assess KOR activation in the mesolimbic reward pathway through GTPγS assays (Park et al., 2000; Sovago et al., 2001; Schroeder et al., 2003; McCarthy et al., 2010). However, there have not been extensive studies that mapped KOR activity profiles throughout the brain specifically during chronic pain. In this study, we determined the brain regions where KORs are highly up-regulated during NP pain, and then determined how KORs in each region contributed to modulating reward and affect in pain.

KOR agonists produce dysphoria and psychotomimesis in humans (Pfeiffer, et al., 1986), and drug conditioned place aversion in rodents (Tajeda, et al., 2013). It has been shown extensively in literature that drug abuse and withdrawal can cause anhedonia and dysphoria

through an increase of dynorphin in limbic system, leading to anxiety and depression (Lindholm, et al., 2000; Frankel, et al., 2008; Isola, et al., 2009; Land, et al., 2009; Solecki, et al., 2009). These forms of anxiety and depression are similar to the mood disorders experienced by chronic pain patients in the clinic. More specifically, rodent inflammatory pain models utilizing plantar formalin or Complete Freund's Adjuvant can cause increase in dynorphin and KOR gene expression, leading to depression in mesolimbic dopamine signaling (Leitl, et al., 2014). Evidence from literature provide inferences that dynorphin and KORs are expressed in medium spiny neurons (MSNs) (Mu, et al., 2011). It is also known that KORs can affect dopamine and glutamate signaling presynaptically (McGinty, 1999). It has been shown that activating KOR can lead to decreases in dopamine levels within the NAc (Di Chiara & Imperato, 1988; Maisonneuve, Archer & Glick, 1994; Ebner, et al., 2010; Rose, et al., 2015). During chronic pain and stress, there is a significant decrease in dopamine levels in the mesolimbic system (Di Chiara, Loddo & Tanda, 1999; Wood, et al., 2007; Geha, et al., 2008; Pais-Vieira, et al., 2009). While the precise pathways have not been fully elucidated, recent studies have alluded to dopamine being modulated by KOR activation within limbic brain regions through activation by synthetic KOR agonists (Tejeda, et al., 2013) or dynorphin antibody in chronic pain states (Suzuki, 2015). Other studies have shown the relationship between KOR expression changes within limbic brain regions and presence of neuropathic pain (Chang, et al., 2014). Chronic pain can also lead to a decrease in opioid- and cocaine-induced dopamine release in the NAc (Taylor, et al., 2015a). Yet, the mechanisms for KOR involvement in the mesolimbic system specifically during chronic NP pain are unclear. This study will investigate the *causal* relationship between KOR expression and dopamine signaling as a factor contributing to reward and mood dysfunction in NP pain.

The stress pathway is highly linked to pain. As described in Chapter 1, chronic pain produces negative affect in patients. Interestingly, KOR activation can lead to negative affect as well, such as anxiety and depressive-like symptoms in both humans and rodents (Land, et al., 2008; Van't Veer & Carlezon, 2013; Hang, et al., 2015). KOR expression and activation in the mPFC can also affect emotional state in the basolateral amygdala (Tajeda, et al., 2015). The amygdala in the brain is highly involved in an animal's response to stress (Badowska-Szalewska, et al., 2015; Wilson, et al., 2015; Aghajani, et al., 2016; Butler, et al., 2016; Patel, et al., 2016). This region is greatly influenced by pain signaling, such that even its physical size can decrease due to chronic pain (Mao & Yang, 2015). Stress and anxiety are highly correlated to fear conditioning in rodents (Elsenbruch & Wolf, 2015; Pavlova & Rysakova, 2015), and contextual fear conditioning can lead to pain hyperalgesia (Labrenz, et al., 2016). It is understood that the fear of pain and the memories of painful experiences are driven by the amygdala in the brain (Simons, 2016). Patients with persistent back pain or fibromyalgia exhibit pain avoidance behavior, such as having guarded movements to prevent pain (Vlaeyen & Linton, 2000; de Gier, Peters & Vlaeyen, 2003; Meier, et al., 2015). These types of behaviors trigger stress and anxiety, which have been highly implicated in pain measurement (Strahl, Kleinknecht & Dinnel, 2000). Dynorphin release at the central nucleus of the amygdala has been hypothesized to facilitate negative affect in chronic pain, which may lead to chronic pain patients to become susceptible to opioid abuse disorder (LeBlanc, et al., 2015). KOR signaling in the amygdala has been heavily implicated in the manifestation of anxiety and depressive-like behaviors (Narita, et al., 2006; Wittman, et al., 2009; Pietrzak, et al., 2014). Corticotropin-releasing factor (CRF) is known to interact with KOR signaling, leading to changes in GABAergic neurotransmission in the central nucleus of the amygdala (CeA) (Crowley & Kash, 2015; Kang-Park, et al., 2015). It is also

highly involved in stress-induced reinstatement of drug seeking in rodents (Funk, Coen & Le, 2014). Thus, studying these behaviors and their signaling pathways in context of chronic pain may lead to new insights to more comprehensive therapeutics beyond the sensory component of pain.

We hypothesized that KOR expression and signaling pathways may be different in males and females, especially in the presence of chronic pain. For example, it is possible that in female mice, basal KOR levels in the amygdala could be higher than in males, and the resulting effects of NP pain may not trigger a significant further increase of KOR activity in this brain region. In studies with MORs in the NAc, it has been shown that females had lower basal MOR activation than in males (Zubieta, et al., 2002). It is possible that if KOR is upregulated in the amygdala of NP pain mice, this upregulation would contribute to stress-induced negative affect behaviors in chronic pain. In this study, we assessed whether KORs are highly activated in the brains of NP pain animals. We expected that our NP pain mice model would develop anxiety and depressive-like behaviors. Furthermore, we predicted that these behaviors were likely caused by KOR activation. To address this hypothesis, we utilized pharmacological tools to manipulate the activity of KORs and subsequently observed behavioral differences between NP pain and sham mice. In addition, our study encompassed sex comparisons for such behavior tests and pharmacological manipulations of KORs.

It is known that chronic stress can lead to CRF signaling that follows stimulation of dynorphin release, thereby activating KORs and produces dysphoria (Bruchas, Land & Chavkin, 2010; Knoll & Carlezon, 2010). Aversion can also arise from a potent activation of KORs pharmacologically, which can lead to JNK signaling (Bruchas & Chavkin, 2010; Ehrich, et al., 2015). The dysphoria caused by activation of KORs through chronic stress mechanisms may be

occurring in chronic pain as well. Based on this concept, our final goal in this study was to link the role of KORs specifically to pain aversion. Thus, we conducted behavior tests to show that KOR activity was a primary driver of the tonic-aversiveness of NP pain.

Methods

Animals

Experiments were performed on adult male and female C57/BL6 mice aged 8-12 weeks postnatal (22-32 g; Sacramento, CA) housed in groups of four per cage. Mice were maintained on a 12/12 h reverse light/dark cycle and were given ad libitum access to food and water. Experiments were carried out during their dark cycle according to protocols approved by the University of California, Irvine Animal Care and Use Committee (IACUC) and in accordance with guidelines set forth by University Laboratory Animal Resources (ULAR).

Neuropathic Pain Model

To initiate the study, I used a peripheral nerve injury model that leads to prolonged NP pain in mice. Animals arrived were housed for 5-7 days to acclimate in vivarium and for handling. I used a chronic constriction injury (CCI), also known as peripheral nerve injury (PNI), as the NP pain model. This model has both construct and face validity with clinical pain and as is commonly described in literature (Mosconi & Kruger, 1996; Pitcher, Ritchie & Henry, 1999; Benbouzid, et al., 2008; Dableh, Yashpal & Henry, 2011; Yalcin, et al., 2014; Taylor, et al., 2014; Taylor, et al., 2015a). Mice were randomly assigned into either sham or PNI surgery groups. Prior to surgery, all animals received acetaminophen (~1 mg) and were subsequently

anesthetized with gaseous isoflurane (~2.5% in O_2). An approximately 1 cm incision was made in the upper left hind-leg. For PNI, the sciatic nerve was constricted with polyethylene tubing (PE20, 2 mm length). The sham group received a similar surgery but without nerve manipulation. A pain naïve control group was also included that did not receive surgery. Von Frey tactile allodynia tests were conducted one day prior to surgery and at multiple time points after surgery. Subsequent weekly Von Frey tests were conducted to confirm chronic pain in the PNI group compared to the naïve and sham groups. All behavioral tests were conducted with experimenter blind to the experimental conditions.

Real-Time Quantitative PCR

Brains were collected from 10 sham and 10 NP pain mice 8-weeks post-surgery and were coronal-sectioned via cryostat (150 µm thick) at -20 °C and mounted on SuperFrost charged slides (Fisher Scientific, Pittsburg, PA). Tissue punches (1 mm diameter) were taken using a disposable biopsy plunger (Miltex, York, PA) for mPFC, NAc, BNST, amygdala (AMYG), hippocampus (HIPP), thalamus (THAL), VTA, and DRN, as these regions were known to express KOR and are involved in either affective or sensory components of pain (Lutz & Kieffer, 2012; Lutz & Kieffer, 2013; Cahill, et al., 2014; Lalanne, et al., 2014). Total RNA was collected from the brain tissue punches via Trizol extraction method (Ambion Life Technologies, Grand Island, NY). RNA was converted to cDNA using 100 U of M-MulV Reverse Transcriptase, 1 µM Oligo d(T)23VN, and 2 mM dNTP mix (New England Biolabs, Ipswich, MA), annealed at 70 °C for 10 min, extended at 40 °C for 1 hr, and inactivated at 95 °C for 5 min. Samples were chilled at 4 °C for 10 min and then stored at -20 °C. Real-time qPCR was conducted using primer sets for dynorphin (DYN), KOR, and beta-actin control genes (Supplemental Table 1).

Using, 96-well optical plates (Applied Biosystems, Singapore), cDNA and PerfeCTa SYBR Green FastMix containing the primer sets (Quanta Biosciences, Gaithersburg, MD) were loaded and run on ABI ViiA7 fast block qPCR machine using cycling conditions in the PerfeCTa SYBR Green FastMix manual. Cycle threshold outputs were calculated and normalized to the actin housekeeping gene to compute ΔCT. Relative expression levels were determined by normalizing sham and NP pain groups to 3 same-age naïve mice brain samples via ΔΔCT method. The results were statistically analyzed using one-way ANOVA with Bonferroni correction between sham and NP pain brain regions.

Primer	Sequence	Bases	Tm (50 mM NaCl)
KOR-F	GCT CCT GGC ATC ATC TGT TG	20	56.2
KOR-R	GCA AAG ACG AAG ACA CAG ATC TTC	24	55.1
DYN-F	TCT AAT GTT ATG GCG GAC TGC	21	58.7
DYN-R	ACT TTT CCT CTG GGA CGC TG	20	60.2
ACT-F	GGC TGT ATT CCC CTC CAT CG	20	62.4
ACT-R	CCA GTT GGT AAC AAT GCC ATG T	22	60.5

Table 4.1: Primer sequences were developed for kappa opioid receptor and dynorphin mRNA used in qPCR experiments. Optimal amplification sequences were determined using Primer-BLAST tool (NCBI), spanning two exons to prevent genomic amplification. The β-actin (ACT-F / ACT-R) sequences used are commonly used and have been validated in literature (Spandido, et al., 2008; Clark, et al., 2008; Liu, et al., 2013; Shi, et al., 2013).

Western Immunoblotting of Phosphorylated KOR

Brains were collected from 4 sham and 4 PNI mice 2-weeks post-surgery and snap-frozen with isopentane at -50 °C, and stored in -80 °C until ready to be sectioned. Brains were coronal-sectioned via cryostat (150 µm thick) at -20 °C, mounted on SuperFrost charged slides, and tissue punches (1 mm diameter) were taken using a disposable biopsy plunger for mPFC, NAc, BNST, AMYG, HIPP, THAL, VTA, and DRN. Tissue punches were homogenized in lysis buffer (50 mM Tris-Base, 4 mM EDTA, pH 7.4) with protease inhibitor cocktail (Pierce Biotech - Thermo Scientific, Rockford, IL), centrifuged at 10000 x g to remove DNA/debris, and

supernatant protein was extracted and stored at -20 °C. Protein samples were mixed with NuPAGE LDS Loading Buffer and reducing reagent (Novex, Carlsbad, CA), heated to 70 °C for 10 min, and stored at -20 °C to be used for gel electrophoresis. Gel electrophoresis of protein samples was conducted using Invitrogen SDS-PAGE gel box, NuPAGE MES Running Buffer and Bis-Tris Mini Gels (Novex, Carlsbad, CA), and loaded alongside PAGE-Ruler Plus Prestained Protein Ladder (Fisher Scientific, Pittsburg, PA), run at 120 V for 2 hours. Proteins from gels were transferred onto nitrocellulose (0.45 µm pore size) (Novex, Carlsbad, CA) using BioRad protein transfer box at 4 °C and 250 mA for 50 min. Membranes were blocked with 5% milk and TBS-T (1%) at RT for 1 hour. Membranes were subsequently incubated with pKOR antibodies made in-house (Bruchas lab, Washington University St. Louis) at 1:1000 dilution in antibody buffer (2% BSA, 2% gelatin from cold-water fish, TBS-T) overnight at 4 °C with gentle shaking. After primary antibody incubation, membranes were washed 3x with TBS-T (10 min per wash) and GOXCH HRP-conjugated anti-chicken secondary antibodies (Novex, Carlsbad, CA) were then incubated at 1:4000 dilution in 5% milk and TBS-T (1%) for 90 min at RT with gentle shaking. The membranes were washed again 3x with TBS-T (10 min per wash), followed by 1 min revelation of membranes using Amersham ECL-Plus substrate (GE Healthcare, Visalia, CA). The membranes were visualized with Li-Cor Odyssey Fc Imager. Membranes were then washed and antibody-stripped using glycine stripping buffer (200 mM glycine, pH 2.6) with shaking at RT for 1 hour, then washed 3x with TBS-T (10 min per wash). Membranes were re-probed with beta-actin antibody (Abcam, Cambridge, MA) with 1:4000 dilution overnight at 4 °C, and subsequent anti-rabbit HRP-conjugated secondary antibody (Life Technologies, Grand Island, NY) with 1:4000 dilution for 1 hour. Band intensities were quantified using Li-Cor Image software and normalized to Beta-actin control bands.

RNAScope In Situ Hybridization

Brains were collected from 6 naïve, 6 sham, and 6 PNI mice 2-weeks post-surgery. Brains were perfused with 4% paraformaldehyde in saline and snap-frozen with isopentane at -30 °C, and stored in -80 °C until further processing. Brains were coronal-sectioned via cryostat (18 µm thick) at -20 °C, and thaw-mounted on SuperFrost charged slides. Custom fluorescent probe labels were designed to fit complementary sequences on mRNA strands for kappa opioid receptor gene (Oprk1) carrying fluorescent AlexaFluor488, tyrosine hydroxylase (TH) carrying Atto555, and glutamate decarboxylase 1 (Gad1) carrying Atto647. Brain slices were incubated with 4% paraformaldehyde and then dehydrated with 50%, 70%, and 100% ethanol washes for 5 min each. Slides were then incubated overnight at -20 °C in 100% ethanol. Slides were then taken out and dried for 5 min and a hydrophobic barrier was drawn around the brain slices. Probes were activated in oven at 40 °C for 10 min. Slides were then incubated with probes following the RNAScope Multiplex processing kit (ACDBiosciences, Newark, CA). Slides were then cover-slipped and sealed with nail polish and stored in dark at -20 °C until visualization. Slides were visualized using a Nikon Ti-E widefield inverted fluorescence microscope and NIS Elements software setup. Image fluorescence intensities were quantified using FIJI (ImageJ) software.

KOR Agonist-Stimulated GTPγS Autoradiography

Brains were collected from 8 naïve, 8 sham, and 8 PNI mice 2-weeks post-surgery. Brains were snap-frozen with isopentane at -30 °C, and stored in -80 °C until further processing. On day of processing, the brains were coronal-sectioned via cryostat (20 µm thick) at -20 °C, and

thaw-mounted on SuperFrost charged slides. Sections were pre-incubated in assay buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, 2 mM GDP, 1 μM DPCPX, pH=7.4) for 15 min. Agonist-stimulated KOR activity was determined by incubating brain sections in [35S]GTPγS (40 pM) with U69,593 (10 μM) for 1 hour at RT. After incubation, slides were washed 2x in ice-cold wash buffer (50 mm Tris-HCl, pH 7.4) followed by a brief wash in ice-cold deionized water (30 sec). Slides were air-dried and exposed to Kodak Biomax film together with [14C] standards for 2 days. Films were developed using Kodak GBX Developer and RapidFix solutions. Films were digitally analyzed and quantified using MicroComputer Image Device (MCID) normalized to the [14C] standard curve, measured in dpm/mg (MCID Imaging Research, St. Catherine, Ontario, Canada). The resulting agonist-stimulated samples were compared to non-agonist-treated brain samples to determine the percent activation of KOR above basal.

Conditioned Place Preference Test

The conditioned place preference (CPP) test is commonly used to determine the sensitization and rewarding effects of drugs in rodents (Schechter & Calcagnetti, 1998; Tzschentke, 1998). The experiment involves classical or Pavlovian conditioning whereby animals are individually placed in an arena containing two chambers, with one chamber paired with a control vehicle (placebo) and the other paired with the drug of interest (Koob, Arends, Le Moal). In our paradigm, we also use a third neutral chamber to further eliminate chamber bias upon initially placing animals into the arena, as well as use the time spent in the neutral chamber as a metric to compare any preference or aversion of both the drug and vehicle treatments. The animals spend several conditioning sessions in one chamber at a time in order to learn which

chamber contains vehicle or drug. Afterwards, the animal is then given access to both chambers (in a drug-free state) and the amount of time they spend in each chamber is recorded. Animals that spend a significantly greater amount of time in the drug-paired chamber are considered positively reinforced and show signs of a preference for the drug. Animals that significantly greater spend time in the control chamber show signs of conditioned place aversion towards the drug. This conditioned place aversion has been validated to be a measure of negative affect (Koob & Volkow, 2010) as well as *acute* pain aversion (Minami, 2009; Navratilova & Porreca, 2014), and generalized opioid receptor blockade has been well-documented to produce this behavior in rodents (Hand, et al., 1988). Our lab uses the three-chamber CPP arena that has a neutral chamber with ~40 lux lighting throughout. For improved discrimination between the two conditioning chambers by our mice, one chamber has soft wire mesh flooring while the other has stiff framed mesh flooring. In addition, one chamber contains spotted walls and the other contains striped walls. Finally, one chamber is cleaned using Virkon's solution and the other is cleaned using 70% ethanol, which leaves lingering distinct odors for the animals to identify each chamber. The conditioning chambers are balanced so that mice spend equal time in both chambers prior to conditioning. In our lab's experiment paradigm, we first perform habituation to the apparatus for two days prior to conducting a precondition test where we record the baseline durations of time each mouse spends in each chamber. Assignment of drug is balanced so drugs are assigned to both stripe and circle chambers and counterbalanced so that only have the mice receive active drug on the first day and the other half receive active drug on the second day of conditioning. Afterwards, mice undergo conditioning over 8 days with either saline or drug for 15 or 30 minutes (depending on drug onset and duration). Each mouse only has access to that one particular chamber to associate the saline or drug with that chamber. Mice are only

conditioned once a day during the dark phase of their light/dark cycle. After conditioning, the mice undergo a post-conditioning experiment whereby they are placed in the arena with access to all chambers, but with no drug or saline treatment. They freely venture into each chamber and the duration of time they spend for each chamber is recorded. If they spend significantly more time in the drug-paired chamber, then they have CPP, and if they significantly spend more time in the saline chamber then they have CPA.

High Performance Liquid Chromatography

Analysis was performed using an Eicom integrated HPLC system (HTEC-500, Eicom Corporation, San Diego, CA) with an Insight autosampler and two Eicom EAS-20s online autoinjectors. Chromatographic separation was achieved using an Eicompak PP-ODS II stationary phase (4.6 mm i.d. x 30 mm, 2-μm particle diameter) and a phosphate buffered mobile phase with 96 mM NaH2PO4 (Fluka #17844), 3.8 mM Na2HPO4 (Fluka #71633), pH 5.4, 1.8-2.5% MeOH (EMD #MX0475-1), 50 mg/L EDTA.Na2 (Fluka #03682), and 500 mg/L sodium decanesulfonate (TCI #I0348) in water purified via a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA). The column temperature was maintained at 20-21 oC. The volumetric flow rate was 350-500 μL/min. Electrochemical detection was performed using an Eicom WE-3G graphite working electrode with an applied potential of +450 mV versus a Ag/AgCl reference electrode. Dopamine (Sigma #H8502) and serotonin (Sigma #H9523) standards were prepared in aCSF (147 mM NaCl (Fluka #73575), 3.5 mM KCl (Fluka #05257), 1.0 mM CaCl2 (Aldrich #499609), 1.0 mM NaH2PO4, 2.5 mM NaHCO3 (Fluka #88208), 1.2 mM MgCl2 (Aldrich #449172), pH 7.3+/-0.03 at room temperature). Standard curves, which were verified daily, encompassed physiological serotonin concentration ranges (0, 7.8, 15.6,

31.2, 62.5, 125, 250, 500, 1000 nM using 20-μL sample volume). The limit of detection was ≤60 amol and the practical limit of quantification was ≤120 amol using a 20-μL sample volume. All dialysate samples for in vivo experiments were collected at 5-min intervals at a dialysate flow rate of 2 μL/min using online EAS-20s autoinjectors. Samples were injected immediately onto the HPLC system.

Probe Insertion

At ZT10-12, each subject was briefly (1-3 min) anesthetized using isoflurane for insertion of a CMA/7 microdialysis probe into the guide cannula. Immediately after insertion, regular aCSF was continuously perfused through the probe at 2 μL/min for 30-60 min followed by a 0.3 μL/min flow rate for an additional 12-14 h to allow recovery from acute neurotransmitter release due to probe insertion.

Morphine-Induced Dopamine Overflow

For all mice, at ZT1:00-2:00 on the day after probe insertion, the aCSF flow rate through the probes was increased to 2 μL/min for 60-120 min prior to collecting dialysate samples for analysis. Basal samples were collected for 60 min, followed by an i.p. injection of 10 mg/kg Morphine. The morphine induced dopamine and serotonin overflow were collected for an additional 250 min.

Open-Field Test

The open-field test was used in this study to compare relative levels of mobility as well as anxiety levels between sham and NP pain mice. Mice that spend less time in the center, open

area of the box are considered more prone to anxiety. A total of 10 sham and 10 NP pain mice were individually placed and monitored for 15 min. Mice were tracked using a video camera and their total time spent in either the perimeter of the arena as well as center of the arena were recorded using EthoVision software. Total distance traveled by each mouse was also recorded.

Marble Burying Test

The marble burying test was used to measure relative levels of repetitive behaviors among mice. An increase in the amount of buried marbles would indicate higher anxiety levels (Nicholas, Kolb & Prinssen, 2006; Jacobson, et al., 2007; Benbouzid, et al., 2008). This test used 20 black glass marbles (1 cm diameter) that were evenly spaced (4x5 matrix) throughout a standard mouse cage with regular bedding. A total of 10 sham and 10 NP pain mice were placed individually in the cages and left alone for 30 min. Afterwards, the buried marbles were counted by at least 3 blinded observers and averaged to give a value for each animal. Marbles were considered buried if the bedding covered at least 2/3 of their surface.

Elevated Plus Maze

The elevated plus maze experiment tests levels of anxiety through risk-taking behaviors in rodents (Carobrez & Bertoglio, 2005). In our study, individual mice were placed on the elevated plus maze apparatus for 5 minutes in a room that had 40 lux light levels. The apparatus contained a set of opposite arms that contained walls and were considered closed spaces that the mice would feel safe to enter, and another set of opposite arms that did not have walls and were open spaces. Using a video camera and EthoVision software analysis, the total time that mice

entered each arm was recorded, as well as the number of times they made the entries into each arm. A total of 10 sham and 10 NP pain mice were tested in this paradigm.

Light-Dark Test

The light-dark test was used in this study to compare relative levels of anxiety between sham and NP pain mice (Yalcin, et al., 2011). Mice that spend less time in the bright, well-lit area of the box are considered more anxious. A total of 10 sham and 10 NP pain mice were individually placed in an arena containing a light compartment (1000 Lumens) and a dark compartment. The mice were left alone for 10 min. and were able to freely roam throughout the arena. Mice were tracked using a video camera and their total time spent in each compartment was recorded using EthoVision software.

Forced Swim Test

The forced swim test is a behavioral paradigm used to model depression in rodents (Yalcin, et a., 2011; Slattery and Cryan, 2012). A total of 10 sham and 10 NP pain mice were individually placed in 4 L beakers filled half-way with water kept at ~28 ^{o}C. The rodents were then filmed swimming for 6 minutes in the water and their mobility was tracked and quantified using EthoVision software during the last 4 minutes of the test.

Splash Test

The splash test is a stress-induced assay that tests grooming behavior; it utilizes the natural tendency mice have to self-groom to remove an irritant (Yalcin, Belzung, Surget, 2008; Yalcin, et al., 2011). A total of 10 sham and 10 neuropathic mice were each sprayed with a 10%

sucrose solution on the dorsal coat of mice. The mice were then monitored for 5 minutes to track their grooming frequency using ethovision software. Mice with elevated depression are expected to have lower grooming frequency when compared to the control group.

Estrous Cycle Monitoring

The procedure for measuring estrous stage of female naïve, sham, and NP pain mice were adapted from McLean et al. (2012) vaginal lavage technique. On the day of estrous cycle testing, mice were taken to behavior room and pre-habituated to the room for ~20 min in approximately 40 lux. Sterile-filtered double distilled water was prepared along with a latex bulb pipetter attached to sterile 200 µl pipette tips. Approximately 50 µl of water was drawn while lifting the mouse out of the cage by the tail. The pipetter was inserted into the opening of the vaginal canal but not penetrating the cervical orifice. The water was expelled and aspirated approximately 2-3 times to collect vaginal cells. The samples were spread on sterile SuperFrost charged slides (Fisher Scientific, Pittsburg, PA) and allowed to air-dry for 2-3 hours at room temperature. After drying, the estrous smears were stained with cresyl violet for ~12 min. The stained slides were then dehydrated with escalating concentrations of ethanol (50%, 70%, 95%, and 100%) for 1 min in each solution. Slides were then fixed in citrus clearing solvent (Thermo Fisher Scientific Inc., Huntington Beach, CA) for 2 min before cover-slip mounting. Cover slips were mounted using DPX histology glue (Sigma-Aldrich Co., Carlsbad, CA). Mounted slides were then air-dried for another 2 days before visualizing samples under a light microscope at 40x magnification to determine estrous stage.

Statistics and Data Analysis

Statistical significance for the Von Frey tactile allodynia test was determined via repeated measures ANOVA with Bonferroni post-hoc correction and $P < 0.05$. Statistical significance for the qRT-PCR experiments at 8-week post-surgery sham and NP pain mice was determined via 2-way ANOVA by surgery group and ipsilateral versus contralateral brain hemispheres, with Bonferroni post-hoc correction and $P < 0.05$. The GTPγS experiments were analyzed via 1-way ANOVA with Bonferroni post-hoc correction comparing either sham or NP pain samples to naïve samples within the same brain region. Significance threshold was set at $P < 0.05$. The same method was used for the GTPγS data involving saline versus CFA-treated rats groups. For the CPP test to U50,488H, 1-way ANOVA with Bonferroni multiple comparisons was used comparing either sham or NP pain mice groups to naïve group's post-preconditioning times, with $P < 0.05$ for statistical significance. For the locomotor test to U50,488H, 2-way ANOVA with Bonferroni multiple comparisons was used comparing either sham or NP pain mice groups to naïve group as well as saline versus U50,488H, with $P < 0.05$ for statistical significance. For the morphine-evoked dopamine microdialysis assay, a repeated measures ANOVA was conducted comparing PNI-JDTic group or PNI-Saline group to Sham-Saline group. Percentage change statistical significance was confirmed to be $P < 0.05$ for all comparisons. DAMGO CPP test was analyzed via 2-way ANOVA with Bonferroni multiple comparisons was used comparing either sham, NP pain, and NP pain with JDTic mice groups as well as saline versus DAMGO, with $P < 0.05$ for statistical significance. Naloxone CPA tests were analyzed with 2-way ANOVA between conditioning drug and surgery condition with Bonferroni corrections within either the wildtype or the PENK-KO mice, and $P < 0.05$ for significance. For the morphine CPP and home-cage CPA in the tonic-aversion to pain paradigm, 2-way ANOVA was conducted between

surgery and drug interactions, and Bonferroni corrections with $P < 0.05$. The negative affect tests were analyzed with Student's t-tests within each behavior test battery, comparing sham and NP pain animals. Statistical significance was set at $P < 0.05$. In the negative affect tests that involved saline or JDTic pretreatment, a 2-way ANOVA was used comparing surgery and drug., with Bonferroni multiple comparisons and $P < 0.05$.

Results

CCI surgery model leads to prolonged tactile allodynia in mice

Prolonged pain may result in transcription of genes that could lead to the subsequent manifestation of negative affect. Prodynorphin, the precursor of dynorphin, may be up-regulated in NP pain mice, which could be responsible for the elevated KOR activation in this pain state. Therefore, I hypothesized that both prodynorphin and Oprk1 could be up-regulated in NP pain mice. Sham and NP pain mice were Von Frey tested over a course of 6 weeks to confirm the PNI surgery was successfully causing chronic pain hypersensitivity. Indeed, NP mice had significantly lower paw withdrawal thresholds (between 0.15 to 0.35) compared to sham animals (Figure 4.1). The slight decrease in paw withdrawal thresholds in the Week 1 time-point for sham animals was likely due to pain caused by the incision made in the hind-leg. Nonetheless, this decrease only lasted for 1 week, and the pain threshold returned to baseline thereafter, while in the NP pain animals the tactile allodynia remained present for at least 6 weeks, which confirms the animals are in a state of chronic pain. Furthermore, there were no sex differences in tactile allodynia, as the PNI surgery induced decreased pain thresholds in both males (Figure 4.2A) and females (Figure 4.2B).

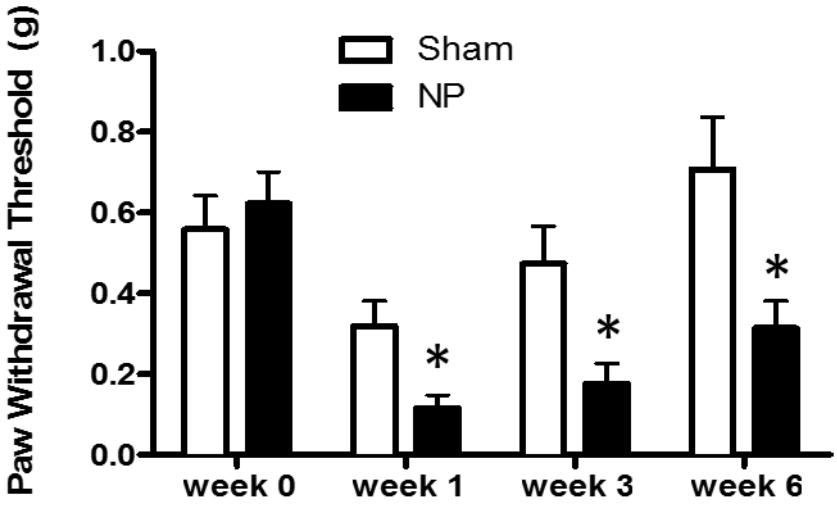

Figure 4.1: Von Frey tactile allodynia test depicts paw withdrawal thresholds (PWT) as the force needed to cause paw retraction approximately half the time. A total of 10 sham and 10 NP mice were tested periodically over a course of 6 weeks. Statistical significance was analyzed via repeated measures ANOVA with Bonferroni post-hoc correction. * = P<0.05

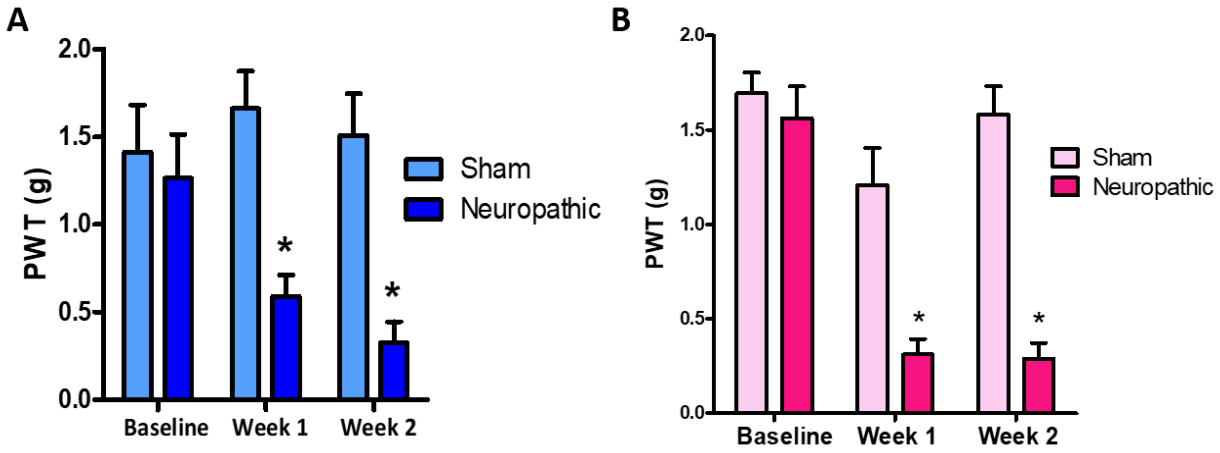

Figure 4.2: Von Frey tactile allodynia test depicts paw withdrawal thresholds (PWT) as the force needed to cause paw retraction approximately half the time. **(A)** Von Frey thresholds for male mice. **(B)** Von Frey thresholds for female mice. A total of 10 sham and 10 NP mice were tested periodically over a course of 2 weeks. Statistical significance was analyzed via repeated measures ANOVA with Bonferroni post-hoc correction. * = P<0.05

Dynorphin and KOR gene expression are elevated in neuropathic pain

Interestingly, both the gene expressions of Pdyn and Oprk1 were significantly up-regulated in the contralateral PFC, NAc, and amygdala in NP pain compared to control animals (Figure 4.3). Oprk1 gene expression was significantly increased in both hemispheres. Contralateral prodynorphin expression in the PFC increased 3.5-fold, NAc increased 10.5-fold, and amygdala increased 5.1-fold in NP pain animals versus sham (Overall ANOVA: $F_{1,140}=39.68$, $p<0.0001$). There were concurrent significant increases in Oprk1 expression in PFC (3.3-fold), NAc (1.4-fold), amygdala (1.6-fold), and VTA (2.3-fold) of NP pain animals compared to sham as well (Overall ANOVA: $F_{1,150}=1250$, $p<0.0001$). The qRT-PCR experiments were also conducted in BNST, hippocampus, thalamus, VTA, and DRN (Table 4.2). There were increases in Oprk1 and Pdyn observed in BNST and VTA, while the hippocampus, thalamus, and DRN did not show any significant gene expression increases in the NP compared to sham. Taken together, these results show that prodynorphin and KOR genes are highly up-regulated in regions involving reward and affect in the NP pain state. It has been shown in that KOR and prodynorphin gene expression can be up-regulated at the spinal cord level in response to long-term pain (Popiolek-Barczyk, et al., 2016; Jiang, et al., 2016). However, my study shows both KOR and prodynorphin gene expression levels are also elevated in the brain, especially in regions that are known to modulate reward and affect. These results indicate the potential role of KOR becoming highly activated in NP pain, which can lead to changes in reward signaling which may result in depressive-like phenotypes.

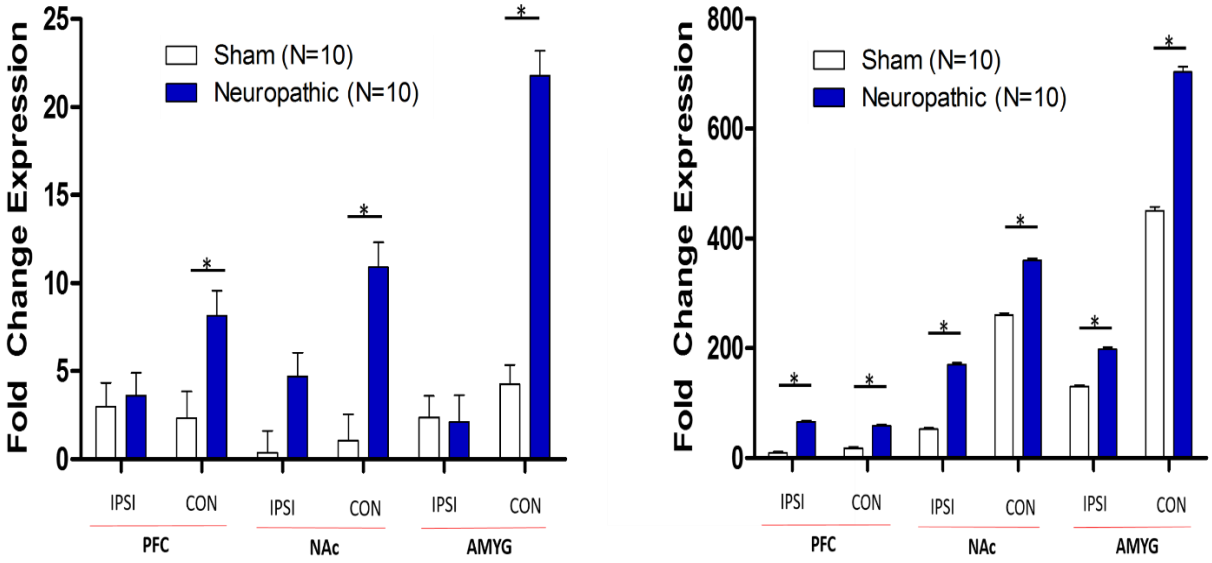

Figure 4.3: Quantified relative Pdyn gene expression (left) and Oprk1 gene expression (right) in different brain regions of 8-week post-surgery sham and NP mice for all qRT-PCR experiments. X-axis includes ipsilateral (IPSI) and contralateral (CON) hemispheres of medial PFC, NAc, and amygdala (AMYG). Statistical significance was analyzed via 2-way ANOVA with Bonferroni post-hoc correction. * = $P<0.05$

a

Oprk1	Sham (N=10)			Neuropathic (N=10)		
	Mean	SEM	N	Mean	SEM	N
PFC-ipsi	9.03	2.46	6	**65.35**	3.01	6
PFC-con	17.53	2.01	6	**58.47**	2.46	6
NAC-ipsi	52.09	2.97	6	**170.54**	3.00	6
NAC-con	260.30	4.16	6	**359.54**	3.88	6
BNST-ipsi	8.53	6.18	6	4.40	2.94	6
BNST-con	83.61	2.77	6	53.67	5.29	6
AMYG-ipsi	130.26	2.72	6	**197.82**	3.65	6
AMYG-con	450.53	7.07	6	**702.98**	10.08	6
HIPP-ipsi	4.41	5.04	6	4.15	3.10	6
HIPP-con	31.37	2.29	6	26.81	3.63	6
THAL-ipsi	3.69	2.52	6	**21.87**	2.02	6
THAL-con	6.97	3.11	6	**61.55**	4.94	6
VTA-ipsi	50.63	5.85	6	**156.49**	4.73	6
VTA-con	76.17	4.90	6	**173.29**	4.94	6
DRN	59.74	6.00	6	38.90	3.18	6

b

Pdyn	Sham (N=10)			Neuropathic (N=10)		
	Mean	SEM	N	Mean	SEM	N
PFC-ipsi	3.00	1.34	6	3.60	1.30	6
PFC-con	2.35	1.50	6	**8.15**	1.42	6
NAC-ipsi	0.38	1.24	6	4.69	1.37	6
NAC-con	1.04	1.52	6	**10.90**	1.42	6
BNST-ipsi	0.72	1.63	6	0.19	1.40	6
BNST-con	3.56	1.18	6	5.60	1.39	6
AMYG-ipsi	2.40	1.19	6	2.12	1.51	6
AMYG-con	4.25	1.12	6	**21.78**	1.42	6
HIPP-ipsi	8.10	1.24	6	2.15	1.34	6
HIPP-con	6.51	1.36	6	4.49	1.59	6
THAL-ipsi	2.20	1.21	6	6.37	1.27	6
THAL-con	6.03	1.13	6	**10.59**	1.31	6
VTA-ipsi	0.29	1.13	6	1.76	1.41	6
VTA-con	2.21	1.09	6	5.54	1.45	6

Table 4.2: Quantified relative Pdyn gene expression **(a)** and Oprk1 gene expression **(b)** in different brain regions of 8-week post-surgery sham and NP pain mice for all qRT-PCR experiments. Statistical significance was analyzed via 2-way ANOVA between ipsilateral and contralateral brain regions and between sham and NP samples, with Bonferroni post-hoc correction. Red highlighted values indicate significant ($P<0.05$) upregulation in gene expression between sham and NP pain brain regions.

Oprk1 is upregulated in dopamine neurons during neuropathic pain

A recent fluorescent in situ hybridization (FISH) method known as RNAscope has become a popular way to gauge RNA levels across entire tissue sections (Wang, et al., 2012; Grabinski, et al., 2015). This method can also be used to generate multi-labeled images of cells expressing a labeled gene, or even co-labeled genes. In particular, prodynorphin gene RNAscope has been successfully conducted recently (Banghart, et al., 2015). In this study, the RNAscope method allowed us to determine which cell types the KOR is primarily expressed within the VTA, as well as both qualitatively and quantitatively ascertain the mRNA changes in NP pain. Based on literature evidence as well as our qRT-PCR results, we hypothesized that upstream dopaminergic projections from the VTA may be affected by KOR activation in the NP pain state. We conducted RNAScope to assess whether KOR gene (Oprk1) expression is specifically increased in dopaminergic neurons. This method allowed for identifying KOR expression among various cell types via colabeling of heterogeneous populations of GABAergic and dopaminergic neurons within the VTA. Figure 4.4 depicts mRNA expression of GAD1 (GABA neurons), tyrosine hydroxylase (dopamine neurons), and Oprk1 genes. PNI animals show elevated expression of Oprk1 compared to sham ($F(2,30)=9.539$, $p<0.001$). Most of this expression is located on TH-labeled neurons, which suggests KOR upregulation may be disrupting dopamine signaling within these cells.

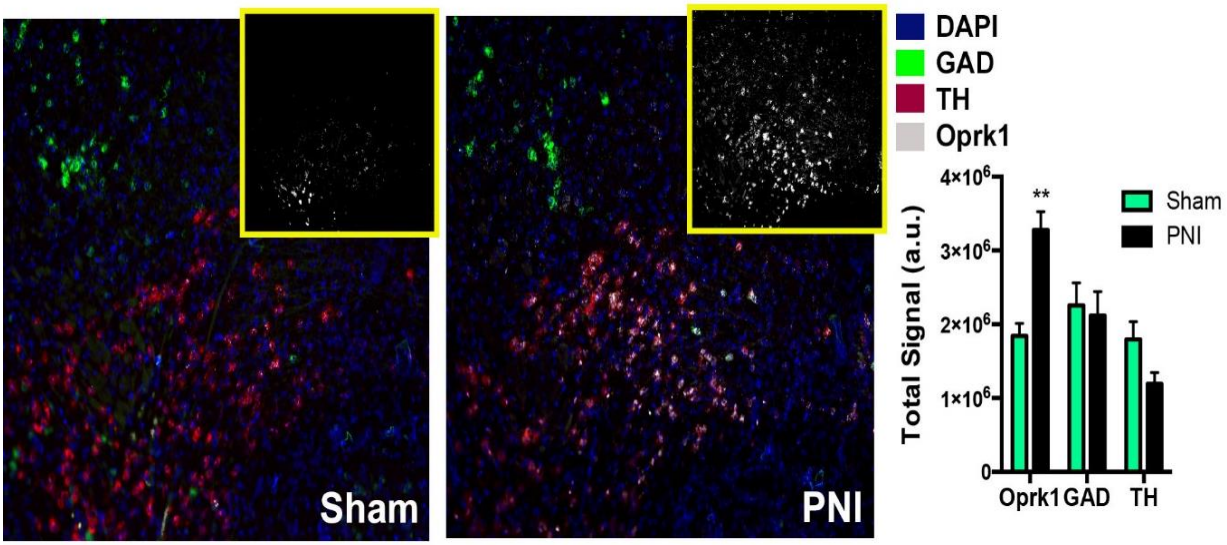

Figure 4.4: Brain slices containing VTA were used for RNAScope multiplex in situ hybridization. Representative VTA images from sham and PNI brain slices are depicted, with blue DAPI, green glutamate decarboxylase, red tyrosine hydroxylase, and white KOR gene. Total fluorescence signals from 6 sham and 6 PNI mice VTA slices were quantified.

KOR is highly activated in brain regions that are involved in regulating anxiety and depression during neuropathic pain

To further verify that KOR is activated in the brain, we conducted a series of Western immunoblotting experiments using a non-commercial phosphorylated kappa opioid receptor antibody (anti-chicken) obtained from Dr. Michael Bruchas's lab in Washington University in St. Louis, MI. Being a G-protein coupled receptor, the KOR becomes phosphorylated upon activation (Chen, et al., 2015). Although not significant, the PFC, NAc, and amygdala showed slight increases in pKOR within NP pain mice brains (Figure 4.5). It was observed, however, significant differences between ipsilateral and contralateral amygdala pKOR expression. This is likely due to the decussation of the pain signals at the spinal cord before reaching the brain,

which may bias the activation of KOR towards the contralateral brain. Overall, these results indicate that KOR activation is potentially greater in the NP than sham animals, especially in brain regions that are important for reward and affect. In conclusion, due to the nature of their localization in the brain and that they are upregulated, KORs should play a role in the negative affect associated with NP pain, which we later investigated.

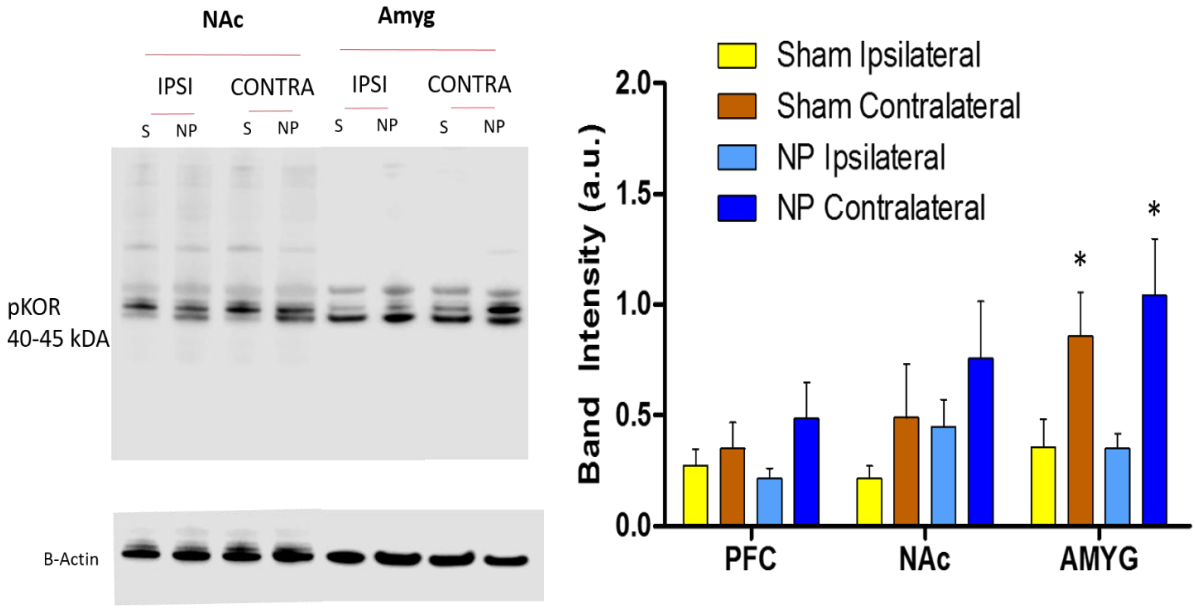

Figure 4.5: Immunoblot of phosphorylated kappa opioid receptor (pKOR) with corresponding actin bands (left) and quantified average pKOR band intensities normalized to β-actin across 4 experiments (right). X-axis includes ipsilateral (IPSI) and contralateral (CON) hemispheres of medial prefrontal cortex (PFC), nucleus accumbens (Nac), and amygdala (AMYG). Statistical significance was analyzed via 2-way ANOVA with Bonferroni post-hoc correction. * = $P<0.05$ compared to same brain hemisphere in sham.

Based on the results from the immunoblots in the previous experiment, we proceeded to identify more specifically where the KOR is activated in order to pinpoint brain sub-regions that are being affected by KOR activation. This can also provide further insight to the possible cell types that are known to be present in brain sub-regions so we may later investigate cell populations that express KOR. Thus, we conducted KOR agonist-stimulated GTPγS autoradiography to map functional activity throughout the entire mouse brain. The results of the GTPγS autoradiography depict significantly increased activation and availability of KORs on cells within the NAc and amygdala of NP pain mice brains compared to their naïve and sham counterparts (Figure 4.6). Furthermore, the NAc core had 17% greater KOR activation and NAc shell had 16% greater KOR activation than sham when both brain sections were stimulated by U69,593. KOR activation in the NAc has been studied in drug intake escalation (Whitfield, et al., 2015). In addition, KOR agonists can prevent the rewarding effects of these drugs (Wee & Koob, 2010). In pain, this reward pathway through the NAc may also be disrupted, as observed with the increased KOR activation in both the core and shell in NP pain mice. Likewise, the BLA showed 11% increase, CeA showed 29% increase, and MeA showed 30% increase in the NP pain brain compared to sham. It has recently been stated that social stress can also increase KOR activation, leading to a decrease in dopamine signaling in the brain (Karkhanis, et al., 2016), with the MeA being a crucial brain region controlling social behaviors (Adams & Rosenkranz, 2015). Because the amygdala plays a critical role in stress and pain-induced memory formation, the activation of KOR in this region may also be contributing to negative affect (Donahue, et al., 2015). It has been shown that activation of KORs in the BLA can inhibit long-term potentiation (LTP) (Huge, et al., 2009), and both the BLA and CeA are crucial to regulating the affective component, and

not the sensory component of pain (Tanimoto, et al., 2003). These results confirm the immunoblot data that KOR is indeed activated during NP pain in the stress and reward pathways.

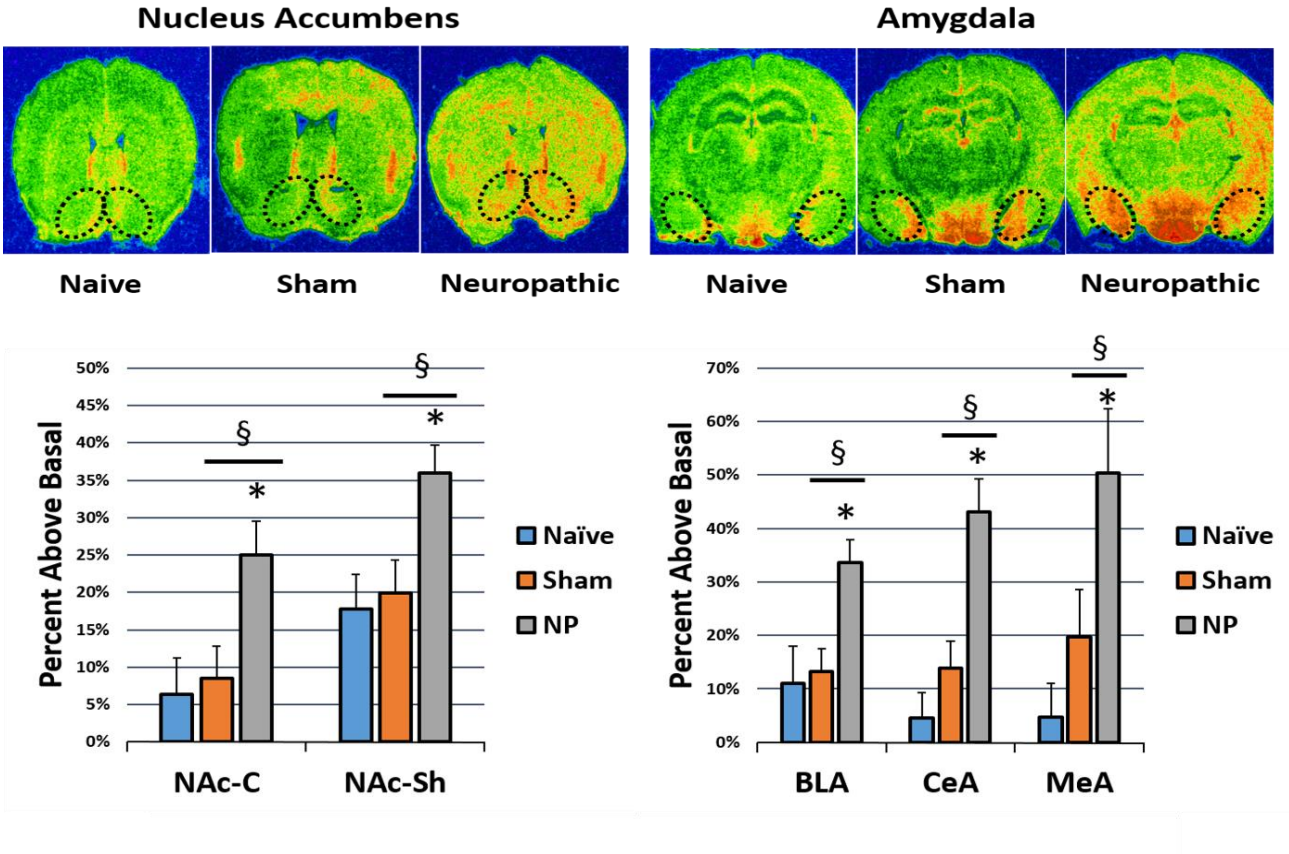

Figure 4.6: Snap-frozen brain sections were used for U69,593 (10 µM) agonist-stimulated GTPγS autoradiography, followed by MCID quantification of KOR activation intensities in various brain sub-regions, expressed in percent above baseline GPCR activity. A total of 8 naïve, 8 sham, and 8 NP pain mice were used for this experiment. NAc core (NAc-C), NAc shell (NAc-Sh), basolateral amygdala (BLA), central amygdala (CeA), and medial amygdala (MeA) were quantified. Statistical analysis was conducted using one-way ANOVA with Bonferroni multiple comparison. * = $P<0.05$ comparing naïve to NP within same brain region. § = $P<0.05$ comparing sham to NP within same brain region.

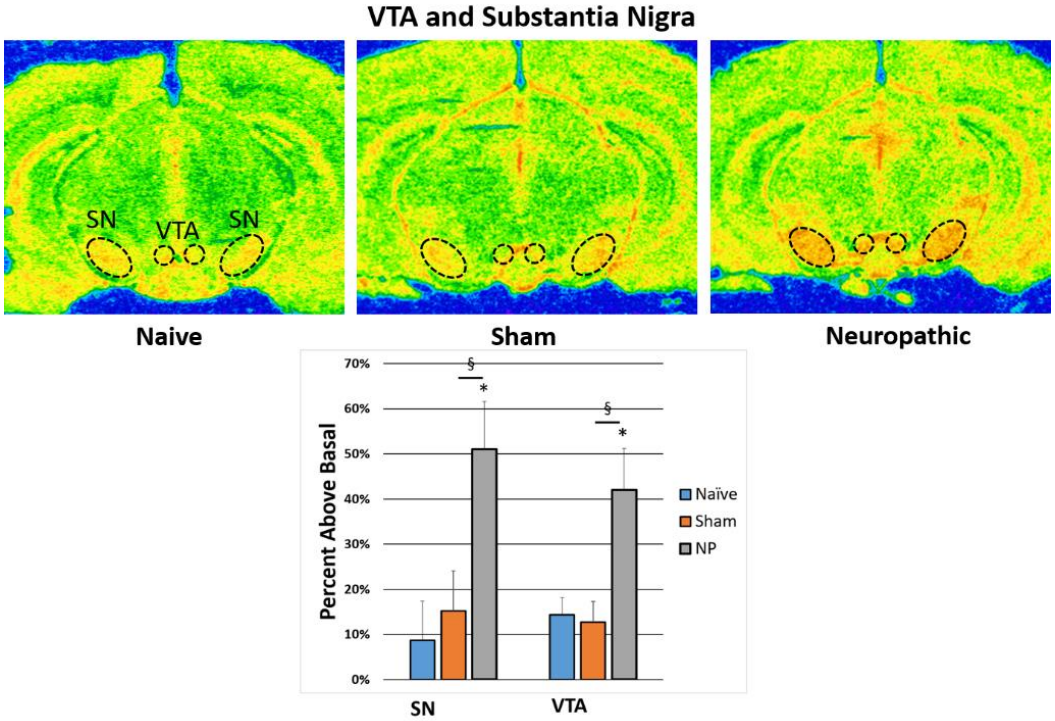

Figure 4.7: Snap-frozen brain sections were used for U69,593 (10 µM) agonist-stimulated GTPγS autoradiography, followed by MCID quantification of KOR activation intensities in various brain sub-regions, expressed in percent above baseline GPCR activity. A total of 8 naïve, 8 sham, and 8 NP pain mice were used for this experiment. Substantia nigra (SN) and ventral tegmental area (VTA) were quantified. Statistical analysis was conducted using one-way ANOVA with Bonferroni multiple comparison. * = $P<0.05$ comparing naïve to NP within same brain region. § = $P<0.05$ comparing sham to NP within same brain region.

Beyond observing the NAc, amygdala, and VTA to compare with our pKOR immunoblot data, we also analyzed other brain regions for KOR activation through GTPγS binding. These regions included the PFC, CPu, BNST, hypothalamus, hippocampus, and raphe nuclei (Figure 4.8). Portions of the PFC such as the cingulate and dorsal peduncular cortex showed significant activation of KOR in NP pain mice compared to naïve counterparts, whereas the infralimbic and prelimbic cortex showed no significant difference (Figure 4.8A). There have been reports that alterations in the reward pathway with chronic morphine treatment can lead to changes in expression of KOR in the mPFC (Yu, Yan & Gong, 2014). KOR in the mPFC is also important for controlling reward and aversion in response to cannabinoid signaling (Ahmad et al., 2013). In addition, dynorphin expression is also increased in response to caustic acid-induced pain (Leitl et al., 2014). Thus, it is likely that pain aversion in the context of our mouse model, is also being mediated via KORs in the PFC. The caudate is involved in various KOR signaling pathways, and greatly implicated in depressive-like behaviors and stress response after repeated swim tests in rodents (Bruchas, Xu & Chavkin, 2008; Reed et al., 2012), as well as adaptive motor and emotional response behaviors in drug dependence (Uriquen et al., 2005). We observed significantly increased activation of KOR throughout the CPu in NP pain mice (Figure 4.8B). The effects of KOR on stress response extends beyond the amygdala brain region throughout the entire hypothalamic-pituitary-adrenal (HPA) axis in the brain. In fact, activation of KOR in the BNST decreases inhibitory GABAergic signals to the amygdala (Li et al., 2012). Our GTPγS binding results showed there is heightened KOR activity in both the anterior and posterior BNST, which suggests a possible facilitation of anxiety in NP pain (Figure 4.8C). Another component of the anxiety pathway involves the hypothalamus. This area is also a hotspot for dynorphin release in the brain (Nakao et al., 1981; Watson et al., 1981; Khatchaturian et al.,

1982). Therefore, it is no surprise that KOR activation may be crucial in this brain region in terms of modulating stress response hormone release such as CRF (Wittman et al., 2009). In our studies, we found a slight but non-significant elevation in KOR functional activity in the LH, as well as a significant increase in KOR activity in the PVH of NP pain mice compared to naïve and sham mice (Figure 4.8D), further confirming the importance of KORs in anxiety behaviors. Referring back to the representative images of Figure 4.6, we can also qualitatively observe such drastic increases in KOR activation within the hypothalamus of the NP pain brain compared to either the naïve or sham brain. Although the hippocampus also plays key roles in stress-based learning and spatial memory, we did not observe changes in KOR activity or bioavailability in all hippocampal sub-regions (Figure 4.8E). This finding may not be surprising, as many other studies regarding chronic drug administration and pain do not show KOR to be a main contributor to hippocampal signaling alterations (Kwon et al., 2008; Li et al., 2010; Guo et al., 2015). The raphe nuclei are responsible for sending serotonergic signals to other regions of the brain in response to stress. Repeated stress can trigger dynorphin and KOR signaling at the raphe regions, leading to dysphoria (Lemos et al., 2012). The DRN also signals to the NAc, which modulates aversion. Blocking KORs specifically in the DRN can prevent KOR-agonist U50488H aversion as well as stress-induced CPP of cocaine (Land et al., 2009). However, in our NP pain mice brains, we observed no differences in KOR activity within the DRN or MRN compared to sham animal brains (Figure 4.8F). This suggests that pain-related stress is probably mediated by the other pathways (previously mentioned) that potentially do not require KOR function in the raphe nuclei.

Overall, these results demonstrate that KOR protein levels are upregulated and becoming available on the cell surface, which upon activation during NP pain is likely to play an active role in contributing to the anxiety and depression.

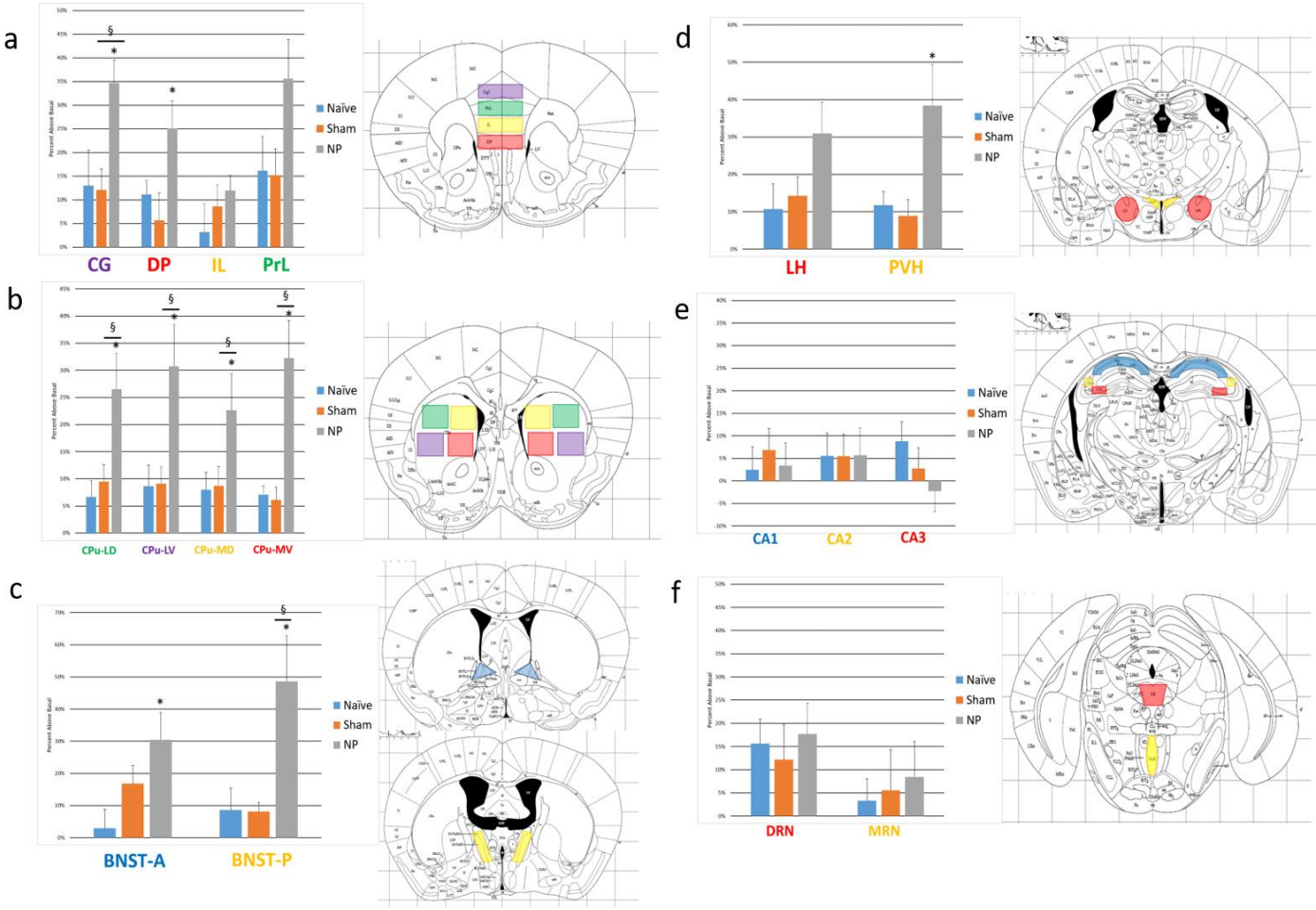

Figure 4.8: Snap-frozen brain sections were used for U69,593 (10 µM) agonist-stimulated GTPγS autoradiography, followed by MCID quantification of KOR activation intensities in various brain sub-regions, expressed in percent above baseline GPCR activity. A total of 8 naïve, 8 sham, and 8 NP pain mice were used for this experiment. **(a)** Cingulate cortex (CG), dorsopeduncular cortex (DP), infralimbic cortex (IL), prelimbic cortex (PrL). **(b)** Caudate putamen lateral-dorsal (LD), lateral-ventral (LV), medial-dorsal (MD), and medial-ventral (MV). **(c)** Bed nucleus of the stria terminalis anterior (BNST-A) and posterior (BNST-P). **(d)** Lateral hypothalamus (LH) and paraventricular nucleus of hypothalamus (PVH). **(e)** Hippocampus CA1, CA2, CA3 neuronal subpopulations. **(f)** Dorsal Raphe (DRN) and medial Raphe (MRN). Statistical analysis was conducted using one-way ANOVA with Bonferroni multiple comparison. * = $P<0.05$ comparing naïve to NP within same brain region. § = $P<0.05$ comparing sham to NP within same brain region.

KORs are highly activated in the reward pathway during chronic inflammatory pain

In the previous sections, we have established that KORs may play a critical role in NP pain. Taking this concept further, we decided that perhaps KOR upregulation may play a more ubiquitous role when it comes to chronic pain. We collaborated with Dr. Jose Moron-Concepcion's lab (Washington University, St. Louis, MO), which worked on Sprague Dawley rats. These rats received injection of Complete Freund's Adjuvant (CFA) (Thermo Fisher Scientific, Waltham, MA), which induced chronic inflammatory pain. The hyperalgesic effects of CFA were confirmed using Plantar Test (Hargreaves method, IITC Life Science). The brains were collected from these rats along with a cohort of saline-treated control rats. Using U69,593-stimulated GTPγS autoradiography, we showed that KOR activity and bioavailability were significantly up-regulated in the CFA-treated rats compared to saline counterparts in the NAc shell (Figure 4.9). JDTic is a highly KOR-selective antagonist (Deehan et al., 2012; Munro et al., 2012; Wu et al., 2012; Chavkin & Martinz, 2015). Co-treatment of U69,593 and JDTic in brain slices of CFA-treated rats showed no increase in KOR binding, which confirms the CFA-mediated increase in signal are distinctively attributed to KOR activation (Figure 4.9).

Thus, it seems that KORs are not only activated in response to NP pain, but also to inflammatory pain. This opioid receptor's function may be integral to chronic pain mechanisms in general, which leads to the idea that perhaps novel therapeutics targeting this receptor may be effective against a range of chronic pain types.

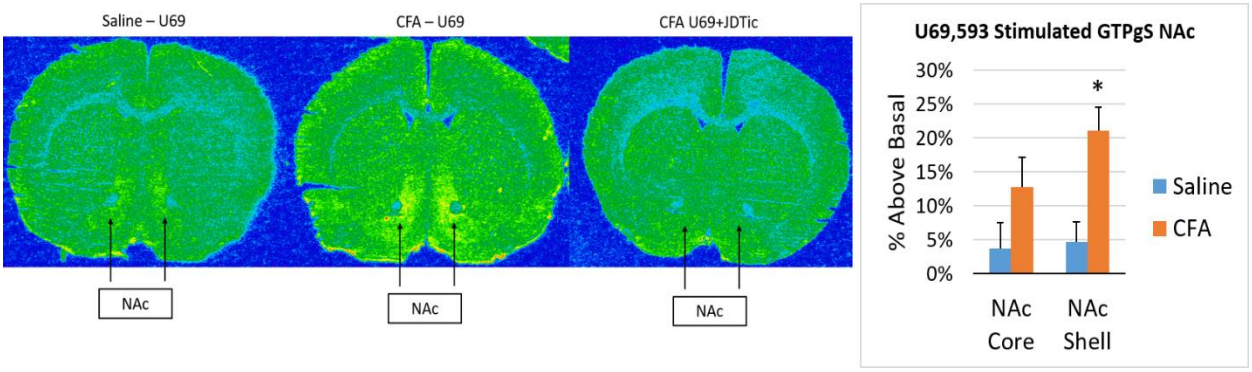

Figure 4.9: Snap-frozen brain sections were used for U69,593 (10 µM) agonist-stimulated GTPγS autoradiography, followed by MCID quantification of KOR activation intensities in various brain sub-regions, expressed in percent above baseline GPCR activity. A total of 8 saline and 8 CFA-treated rats were used for this experiment. Replicate slices were taken from the same 8 CFA animals to conduct the U69,593+JDTic antagonist control assay. NAc core (NAc-C) and NAc shell (NAc-Sh) were quantified. Statistical analysis was conducted using one-way ANOVA with Bonferroni multiple comparisons. * = $P<0.05$ comparing saline to CFA treatment within same brain region.

Kappa opioid receptor signaling is sensitized in neuropathic pain

It has been postulated that KOR signaling may be highly involved in pain aversion, as many of the pathways mediating aversion overlap with pain and reward pathways within the brain (Cahill et al, 2014). Thus, we sought to identify whether KOR signaling pathways were highly activated in the context of chronic pain. Using the conditioned place preference paradigm, 12 naïve, 12 sham, and 16 PNI male animals were given subcutaneous injection of either saline vehicle or U50,488 (5 mg/kg) in alternating repetition over 8 conditioning days (Figure 4.10A). After conditioning, the animals were recorded in terms of time spent in each drug-paired chamber during the post-conditioning session. The PNI animals showed significantly greater conditioned place aversion compared to naïve or sham counterparts, suggesting that male animals in NP pain have heightened sensitivity to the aversive effects of KOR agonist. Sex differences were observed in this experiment, as both sham and PNI female mice developed

place aversion to U50,488 (Figure 4.10B). This may be due to sham surgery effects that could still present with sensitized KOR function even after 2 weeks post-surgery.

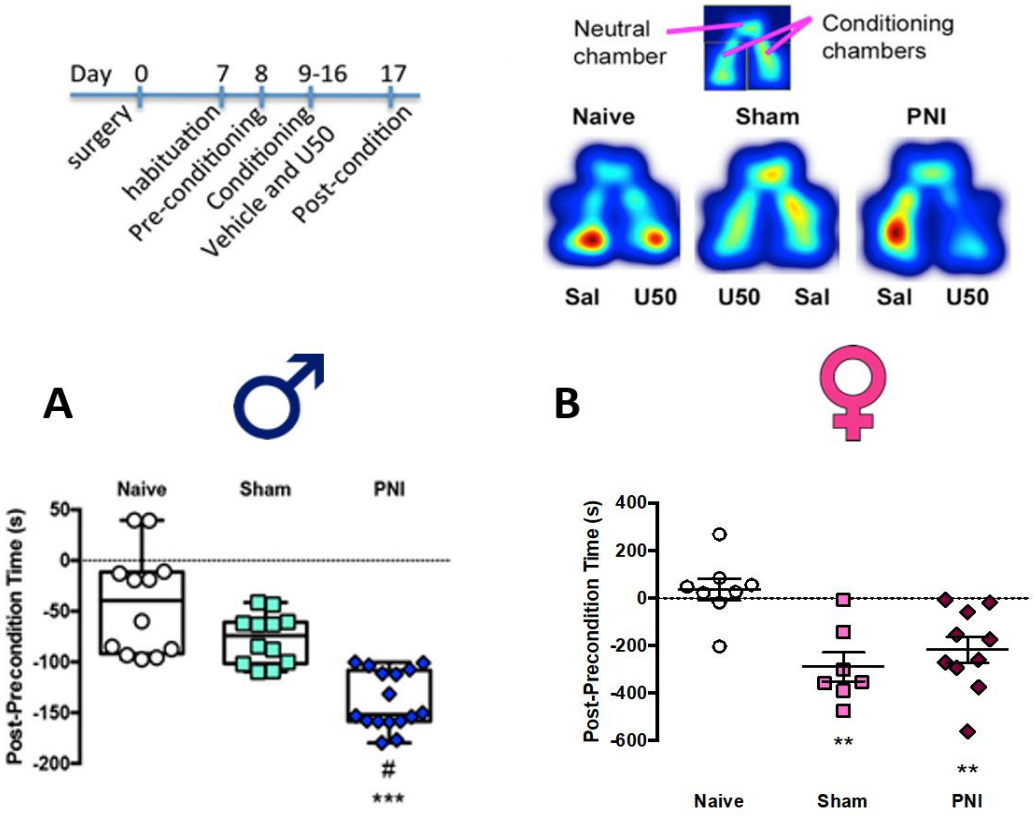

Figure 4.10: KOR signaling is sensitized in NP pain. Treatment and conditioning timeline are depicted along with representative heatmap traces of each surgery group. Data are expressed as median with 25% and 75% quartiles, minimum and maximum values with all data points included.
(A) 12 naïve, 12 sham, and 16 PNI male mice were used in U50,488H conditioned-place aversion 3-chamber paradigm. Subthreshold dose of KOR agonist U50488H (U50,488H, 5 mg/kg, i.p.) did not exhibit significant place aversion in naïve or sham animals, but drastically produced place aversion in PNI male mice. A one way ANOVA revealed a significant effect ($F_{(2,37)}$=26.19), ***p<0.001. #p<0.05 compared to sham, ***p<0.001 compared to naïve.
(B) 8 naïve, 7 sham, and 10 PNI female mice were in U50,488H conditioned-place aversion 3-chamber paradigm. Subthreshold dose of KOR agonist U50,488H (U50,488H, 5 mg/kg, i.p.) exhibited significant place aversion in sham animals and PNI female mice, but not naive. A one way ANOVA revealed a significant effect ($F_{(1,38)}$=8.123), **p=0.007. There was no significant difference between sham and PNI female mice treated with U50,488H

Mechanisms of KOR turnover are accelerated in neuropathic pain

Activating KORs via selective full agonists can lead to decreased locomotor activity in rodents (Castellano & Pavone, 1987; Castellano et al., 1988; Leyton & Stewart, 1992). We validated this effect in our study with naïve and sham animals (Figure 4.11). Moreover, we observed this hypolocomotor activity was also present in PNI animals at the same level. In addition, we noted that both males (Figure 4.11A) and females (Figure 4.11B) showed U50,488H-induced hypolocomotion compared to their respective saline-treated counterparts. Next, we measured locomotor activity of naïve, sham, and PNI male mice given either saline or U50,488H at both the 6th and 11th days post-surgery (Figure 4.12A). However, all groups in this experiment were given a single dose (10 mg/kg) i.p. injection pretreatment of KOR antagonist JDTic. Because JDTic is a long-acting KOR antagonist (Deehan et al., 2012; Munro et al., 2012; Wu et al., 2012; Chavkin & Martinz, 2015), this single dose was expected to negate the hypolocomotor effects of U50,488H. In naïve and sham male animals at 6 days post-surgery, U50,488H had no effects on locomotor activity, which was expected due to the JDTic pretreatment effect. However, the agonist treatment significantly decreased locomotor activity in the PNI male animals. The effect was tested again at 11 days post-surgery and we observed a more drastic decrease of locomotor activity in PNI male animals. This phenomenon also became noticeable in sham male animals at 11 days post-surgery, likely due to the diminished effect of JDTic at this later time point. These results demonstrate enhanced KOR sensitization and signaling in NP pain, which is driving the aversion and dysphoria pathways in male animals. In female animals, this effect was not the case. U50,488H did not induce hypolocomotion in both sham and PNI female mice at Day 6 (Figure 4.12B), but by Day 11, sham and PNI female mice both showed significant hypolocomotion. Taken together, it seems that female mice may not

have the quick KOR cycling mechanism during the PNI state that the males possess. These results may explain possible behavior differences and pain coping mechanisms between males and females.

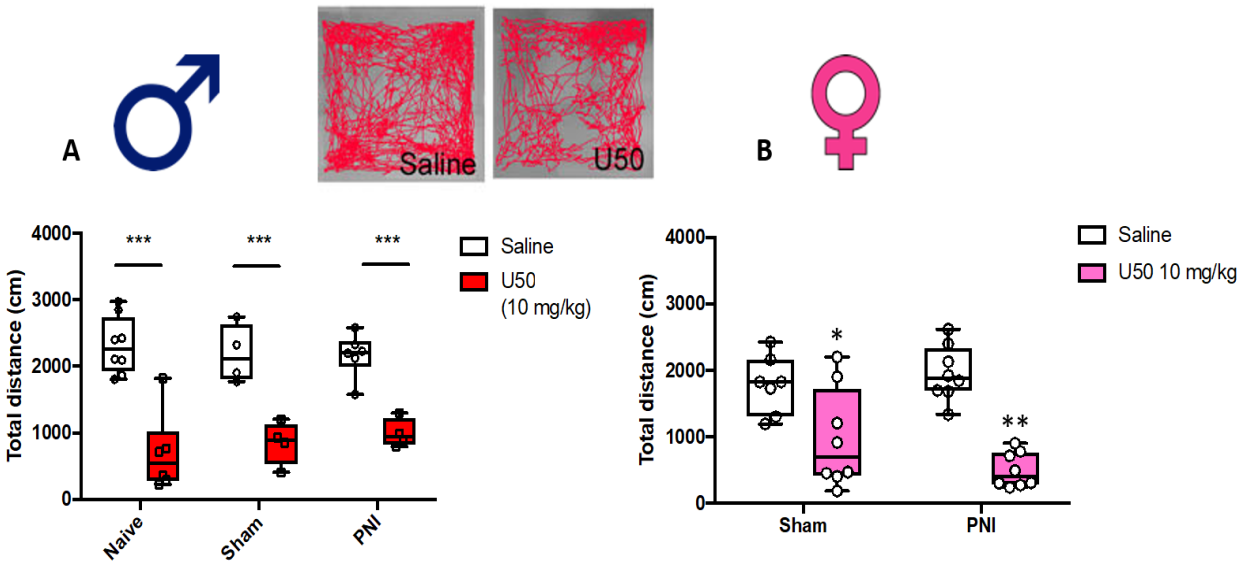

Figure 4.11: Approximately half of the animals from each surgery group were given saline vehicle and the other half were given U50,488H. Their total distance traveled and mean velocities were recorded for each of the days. Representative activity tracks are presented comparing saline versus U50,488H alongside their respective surgery groups. Data are expressed as median with 25% and 75% quartiles, minimum and maximum values with all data points included. There was no significant difference between saline-injected naïve, sham and PNI mice in both male and female groups.
(A) Locomotor activity in an open-field arena for 8 naïve, 8 sham, and 8 PNI male mice were measured. Two way ANOVA revealed a significant effect of treatment ($F_{(1,26)}$ = 78.88, p<0.0001), but not surgery ($F_{(1,28)}$=0.82, p=0.4527). ***p<0.001 compares saline to U50,488H. N=8 per group.
(B) Locomotor activity in an open-field arena for 16 sham and 16 PNI female mice were measured. Two-way ANOVA with Bonferroni post-hoc analysis revealed an effect of treatment ($F_{(1, 28)}$=13.19, p=0.0011) but not surgery ($F_{(1,28)}$=0.08, p=0.7855). *p<0.05 and **p<0.01 both compare saline to U50,488H. N=8 per group.

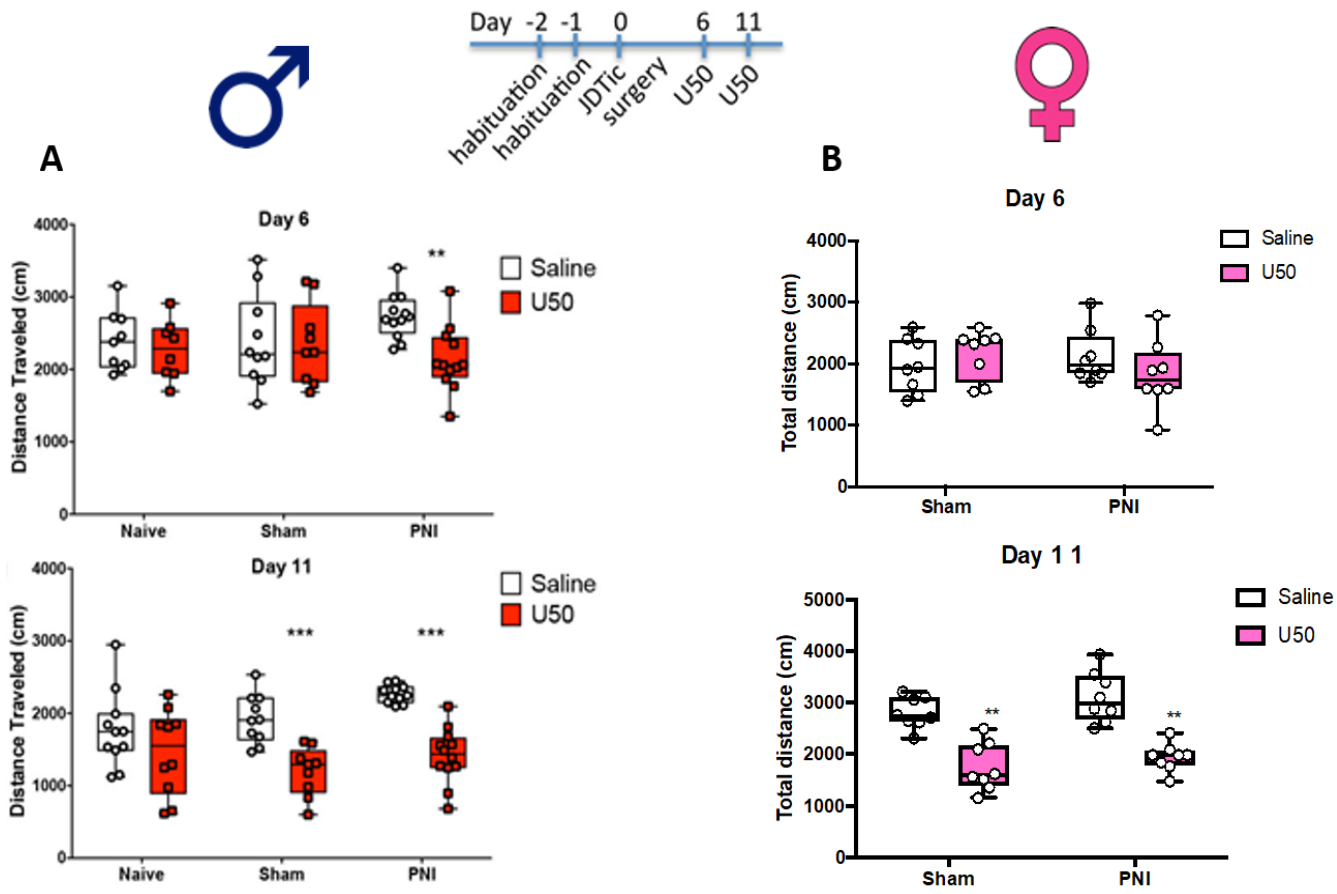

Figure 4.12: KOR turnover is increased in NP pain males. All animals were given pre-treatment of KOR-selective antagonist, JDTic. Approximately half of the animals from each surgery group were given saline vehicle and the other half were given U50,488H at 6-days and 11-days post-surgery. Their total distance traveled and mean velocities were recorded for each of the days. Day 11 presentative activity tracks are presented comparing saline versus U50,488H alongside their respective surgery groups. Data are expressed as median with 25% and 75% quartiles, minimum and maximum values with all data points included. There was no significant difference between saline-injected naïve, sham and PNI mice in both male and female groups.
(A) Locomotor activity in an open-field arena for 17 naïve, 19 sham, and 24 PNI male mice were measured. Two way ANOVA of Day 6 data revealed a significant effect of treatment ($F_{(2,54)}$ = 4.33, $p<0.05$), but not surgery ($F_{(2,54)}$=0.259, p=0.773), and there was no significant interaction ($F_{(2,54)}$=2.258, p=0.114). Thus, there was no significant different between saline-injected naïve, sham and PNI mice. **p<0.01 compares saline to U50,488H. N=8-12 per group. Two way ANOVA of Day 11 data revealed a significant effect of treatment ($F_{(2,57)}$ = 36.67, $p<0.001$), but not surgery ($F_{(2,57)}$=0.2.82, p=0.068), and there was no significant interaction ($F_{(2,57)}$=2.63, p=0.081). ***p<0.001 compares saline to U50,488H.
(B) Locomotor activity in an open-field arena for 16 sham and 16 PNI female mice were measured. Two way ANOVA of Day 6 data revealed no significant effect of treatment ($F_{(3,28)}$ = 1.53, p=0.2286). N=8 per group. Two way ANOVA of Day 11 data revealed a significant effect of treatment ($F_{(3,28)}$ = 32.38, $p<0.001$). **p<0.001 compares saline to U50,488H.

Kappa opioid receptor activation mediates decreased dopamine signaling during neuropathic pain

We have now established that dynorphin and KOR gene expression is present in the NAc. It is also well-understood that mesolimbic signaling pathways involving the NAc and VTA regulate mood and reward (Berridge & Kringelbach, 2013 & 2015; Zarrindast & Khakpai, 2015). In addition, KORs are known to regulate these pathways (Ehrich et al., 2015; Chartoff et al., 2016). Our lab has previously shown opioid-evoked dopamine release is blunted in NP pain compared to control sham animals (Taylor et al., 2015a). Because chronic pain is known to influence dopamine signaling between the NAc and VTA (Schifirnet, Bowen & Borszcz, 2014; Lee, et al., 2015; Sagheddu, et al., 2015; Taylor, et al., 2015a), the next step was to determine whether KORs are directly playing a role in mediating dopamine release especially in the NP pain state. It has been extensively shown that the presence of KOR activation at the VTA can inhibit dopamine signaling from this region to the medial PFC (Margolis, et al., 2003; Margolis, et al., 2005). The VTA also has major projections to the NAc and BLA, whereby KORs can also play modulatory roles (Ford, Mark & Williams, 2006). In accordance with the effects of KORs on dopamine release (Sun, 2011; Bruijnzeel, 2009; Lalanne, et al., 2014), we postulated that KOR activation maybe driving mood and emotion dysfunction in PNI animals. We performed a morphine-induced dopamine microdialysis study in the NAc of 8 sham, 8 PNI, and 8 JDTic-pretreated PNI mice (Figure 4.13). Morphine is known to trigger dopamine release at the NAc, which was observed in the sham animals. However, morphine was not able to induce dopamine release in PNI animals. When we pre-treated PNI animals with the KOR-selective antagonist JDTic, we morphine-induced dopamine release was restored to similar levels as the sham group.

These results show that KORs inhibit dopamine release during NP pain, which may represent a possible mechanism for reward pathway disruption.

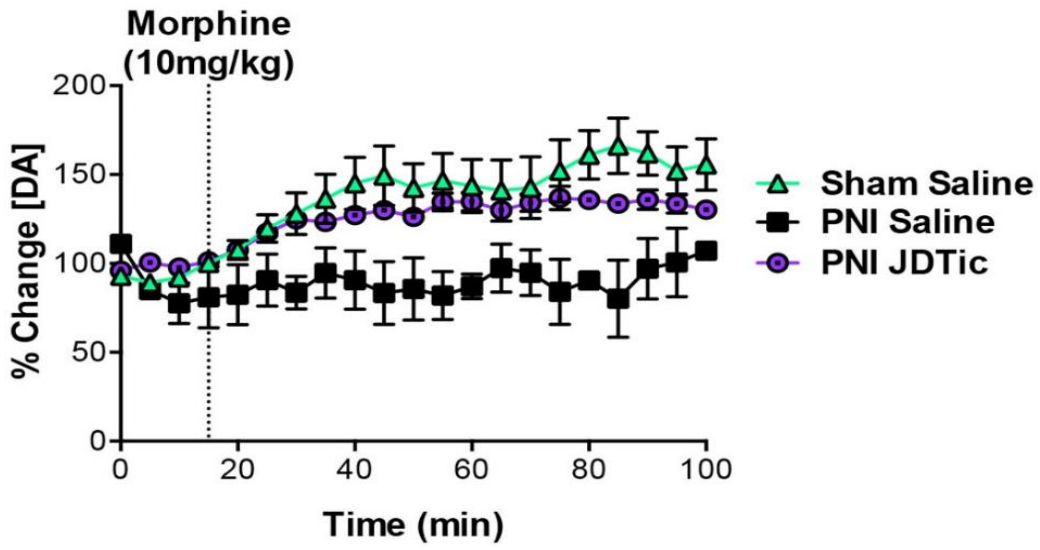

Figure 4.13: Kappa opioid receptor activation is involved with decreased dopamine signaling during NP pain. Microdialysis of evoked dopamine release over time was measured as percent change from baseline following systemic morphine injection in sham, PNI, and JDTic-pretreated PNI mice.

Kappa opioid receptor upregulation plays a role in the dysfunction of the reward pathway during neuropathic pain

To determine the effects of KOR upregulation on the reward behavior of NP pain mice, we used a CPP paradigm (Figure 4.14). We performed intra-VTA injection of the mu-opioid agonist, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), as a pharmacological tool because it is known to produce CPP (McBride, Murphy & Ikemoto, 1999; Narita et al., 2010). To test whether KORs were involved in regulating the reward circuitry under the CPP paradigm, we also divided all surgical groups into either JDTic or non-JDTic pre-treatment groups to isolate the effects of KORs in this paradigm. Approximately one-week post-surgery, we habituated animals to the CPP chambers, and then began pre-conditioning tests a day later. Pre-condition inherent bias from the mice were counterbalanced based on surgical group, chamber pairing to drug, and alternating dates that each animal were to be given vehicle or drug. We performed a total of 8 conditioning days (4 days vehicle and 4 days DAMGO) followed by the post-conditioning day. As expected, we observed that sham animals, regardless of JDTic pre-treatment, all showed significant CPP to DAMGO (Figure 4.14). However, PNI animals did not show any CPP to DAMGO, which suggests that the reward pathway is disrupted during the NP pain state. Remarkably, a JDTic pretreatment to PNI animals was able to restore CPP to DAMGO, which demonstrates a role of KORs on interrupting the reward pathway, but only in the presence of NP pain. Taken together, these results demonstrate that KORs are upregulated in the reward pathway and is likely playing an active role in the dysphoria of PNI animals.

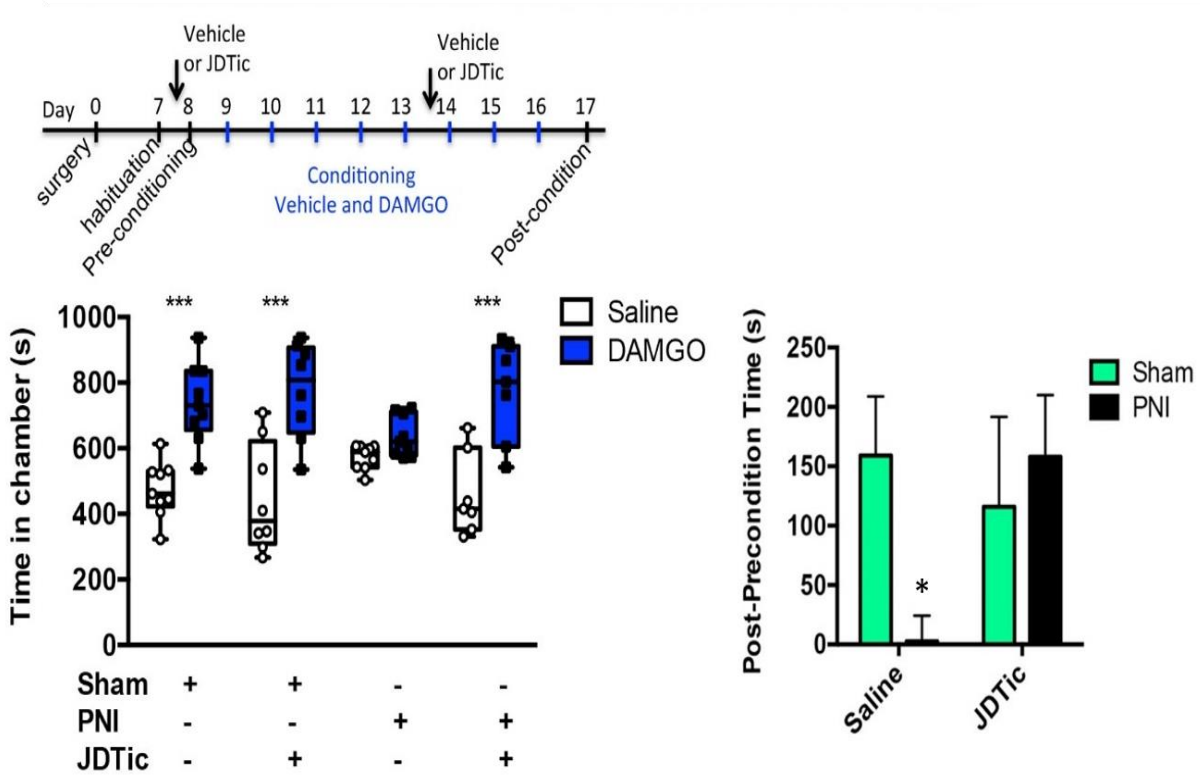

Figure 4.14: Kappa opioid receptor upregulation plays a role in the dysfunction of the reward pathway during NP pain. Intra-VTA DAMGO (1ng/bilateral injection) conditioned-place preference was conducted on sham versus PNI mice, with half the mice from each surgery group given pretreatment of KOR-selective antagonist, JDTic. Results are displayed as total time spent in either saline or DAMGO-paired chamber for each treatment and surgery group. Total increase in time spent between precondition versus postcondition for the DAMGO-paired chamber of each treatment and surgery group is also depicted. Intra-VTA DAMGO produced a CPP in sham but not PNI mice. Pretreatment of mice with KOR antagonist JDTic (10 mg/kg, i.p.) did not alter DAMGO CPP in sham animals but recovered DAMGO CPP in pain animals. Data are expressed as median with 25% and 75% quartiles, minimum and maximum values with all data points included, N=8-9 per group. Two way ANOVA revealed a significant effect of surgical group ($F(1,58)=71.7$, $p<0.001$ and a significant interaction ($F(3,58)=4.82$, $p<0.01$ but not of drug ($F(3,58)=0.029$, $p=0.993$). ***$p<0.001$ compares saline to DAMGO. *$P<0.05$ in post-precondition time between sham and PNI. Data are also presented as reward value (post condition time – precondition time in the drug paired chamber).

The tonic aversive component of chronic pain is driven by kappa opioid receptor activation

Naloxone (NLX) is a non-specific opioid receptor antagonist (Codd et al., 1995). Naloxone is known to cause aversion in rodents (Dymshitz & Lieblich, 1987) and humans (Kobrinsky et al., 1988). To specifically determine the effects of KOR antagonists and CPA in the PNI mouse model, we used cohorts of either wild-type or proenkephalin knockout (Penk-KO) mice. Penk-KO animals lack the ability to produce the endogenous ligand, enkephalin, which activates mu- and delta-opioid receptors. This model was used to eliminate inherent activity of the other opioid receptors in the CPA paradigm in order to enhance the selectivity of naloxone towards the KOR system. We chose this paradigm because the JDTic could not be used as a direct pharmacological tool to gauge KOR effects on CPA since it was long-acting, and the animals would not readily associate the JDTic with the conditioning chamber. In wild-type (surgery-naïve) mice, we confirmed that naloxone produces conditioned place aversion (CPA); this effect was not due to blocking of KORs because animals given JDTic pretreatment also showed the same aversive response (Figure 4.15A). However, wild-type PNI animals showed a significant aversion to naloxone, with even more profound aversion in PNI animals that were pretreated with JDTic. Interestingly, we observed no CPA in NLX-treated naïve Penk-KO animals and a remarkable CPP in PNI animals, suggesting that the antagonism by NLX selectively on KORs blunted aversion (4.15B). JDTic had no effect on NLX CPA in Penk-KO animals, and even blocked the NLX CPP in PNI animals (Figure 4.15C). These findings confirm that NLX was indeed targeting KORs to produce the CPP in Penk-KO PNI animals because JDTic pre-blocked the aversive effects of KORs in these animals and removed the conditioned association of NLX to the drug-paired chamber. In summary, heightened activation of KORs in PNI mice cause and aversive state can be blocked with NLX.

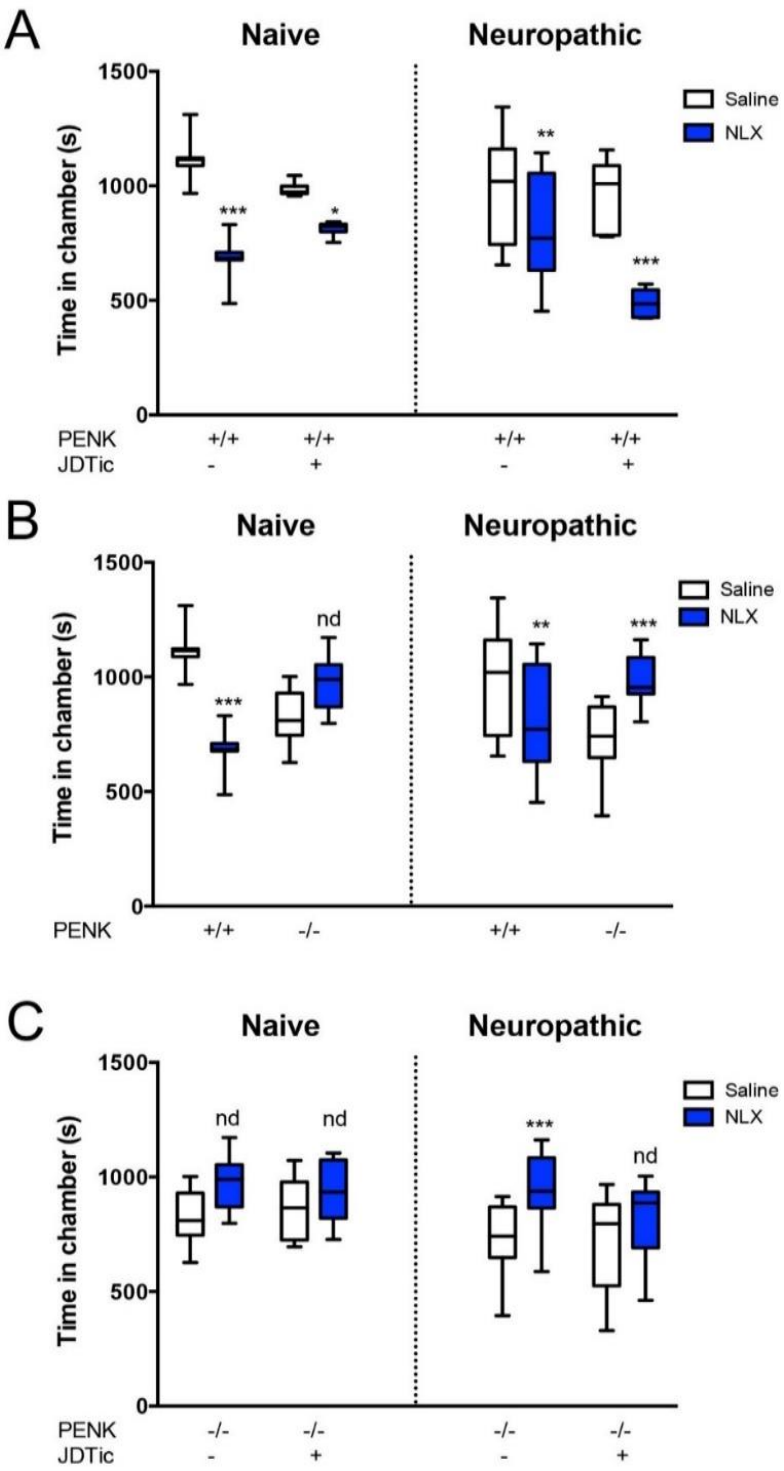

Figure 4.15: Naloxone aversion is blunted in NP pain due to blockade of KOR-mediated aversion **(a)** Naloxone conditioned place preference of wildtype naïve versus PNI mice was conducted, with half of the animals in each surgery group given a pretreatment of KOR-selective antagonist, JDTic. **(b)** Naloxone conditioned place preference comparing wildtype and proenkephalin (PENK) knockout naïve versus PNI mice was conducted. **(c)** Naloxone conditioned place preference of PENK knockout naïve versus PNI mice was conducted, with half of the animals of each genotype given a pretreatment of KOR-selective antagonist, JDTic.

In naïve mice: Naloxone (NLX) produced a place aversion (CPA) in wild type mice, but naloxone CPA was absent in pro-enkephalin (PENK) knockout mice. In chronic pain mice: Naloxone produced a CPA in wild type mice, but produced a place preference (CPP) in PENK knockout mice. Two-way ANOVA revealed a significant effect of genotype ($F(1,82)=7.971$, $p<0.01$) and a significant interaction ($F(3,82)=56.43$, $p<0.001$) but not of drug ($F(3,82)=0.729$, $p=0.537$). **p<0.01, ***p<0.001 compares saline to naloxone. In PENK knockout mice, administration of the KOR antagonist JDTic (10 mg/kg, i.p.) prevented naloxone CPP in chronic pain animals (data highlighted in box). Two way ANOVA revealed a significant effect of conditioning drug ($F(1,80)=14.11$, $p<0.001$) and a significant effect of surgical condition ($F(3,80)=3.098$, $p<0.05$) but not an interaction ($F(3,80)=0.722$, $p=0.542$). ***p<0.001 compares saline to naloxone. Data are expressed as median with 25% and 75% quartiles, minimum and maximum values with all data points included, N=5-18 per group.

To provide further evidence that KORs contributed to the tonic-aversive component of chronic pain, we performed two additional experiments. Previous research reported that analgesic treatment prevents the expression of CPP (negative reinforcement) to a nerve block and other analgesic drugs in chronic pain animals due to the absence of motivation to seek pain relief (Navratilova, Atcherley, and Porreca, 2015). Here we show that JDTic completely blocks expression of morphine CPP in chronic pain, but not sham animals (Figure 4.16B). Thus, alleviation of the tonic-aversive component of chronic pain by KOR blockade was sufficient to prevent expression of morphine CPP in pain but not sham animals, because the primary motivation for morphine CPP was relief from the tonic aversive component of pain. The second experiment took advantage of a conditioning paradigm where animals were conditioned to only one chamber of the CPP apparatus beginning 7 days post-injury. We show that chronic pain, but not sham, mice showed a place aversion to the conditioning chamber demonstrating the going tonic-aversive component of pain (Figure 4.16D). In a separate cohort of mice, administration of JDTic 24h prior to the beginning of conditioning prevented this place aversion. Taken together, these results provide strong evidence that KORs contribute to the tonic-aversive component of chronic pain.

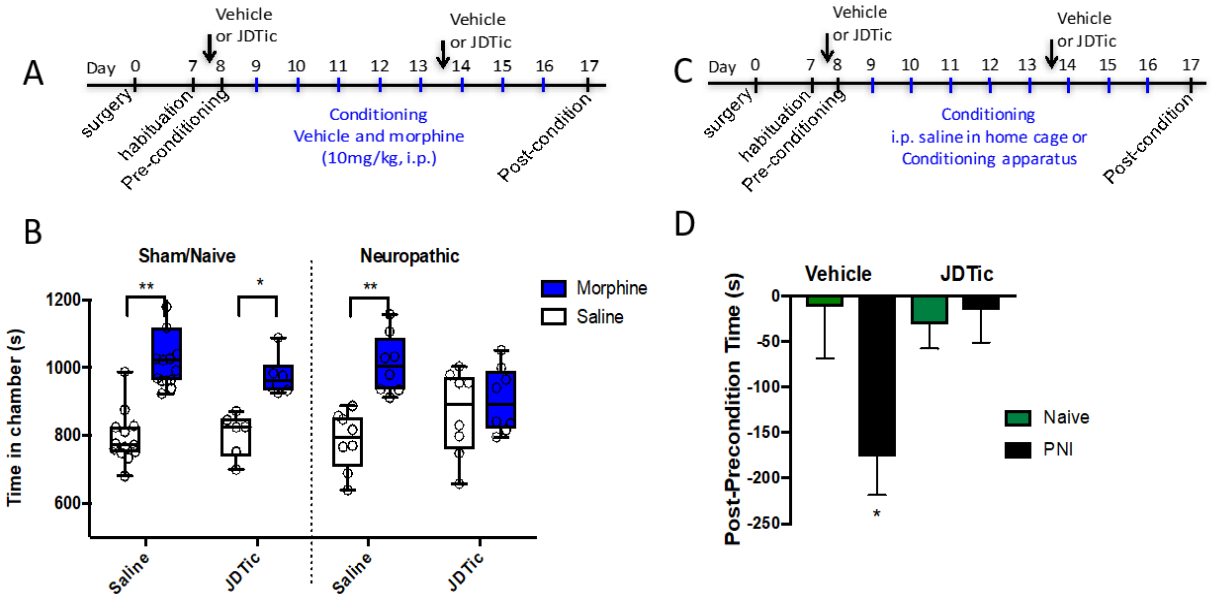

Figure 4.16: The tonic aversive component of chronic pain is driven by kappa opioid receptor activation. (A) Time course of treatment paradigm. (B) Morphine produced a place preference (CPP) in sham mice that was not altered by pretreatment with the KOR antagonist JDTic (10 mg/kg, i.p.) however, place preference to morphine was blocked by JDTic in chronic pain mice. Two way ANOVA revealed a significant effect of surgery ($F_{(1,64)}$=49.13, p<0.001) and a significant interaction ($F_{(3,64)}$=4.214, p<0.01) but not of drug ($F_{(3,64)}$=0.590, p=0.624). *p<0.05, **p<0.01 compares saline to morphine. (C) Time course of treatment paradigm where mice received saline (i.p.) injections daily in home cage or conditioning chamber. (D) Sham animals conditioned to CPP apparatus did not show place aversion or preference to chamber that they received saline but chronic pain mice developed an aversion to the conditioning chamber. This aversion demonstrates ongoing tonic aversive component of chronic pain. Treating mice with KOR antagonist JDTic (10 mg/kg, i.p.) prevented this aversive state. Two way ANOVA revealed a significant effect of surgery ($F_{(1,28)}$=4.343, p<0.05) and a significant interaction ($F_{(1,28)}$=4.343, p<0.05) but not of drug ($F_{(1,28)}$=2.55, p=0.121). *p<0.05 compares sham to PNI. Data are expressed as (A) median with 25% and 75% quartiles, minimum and maximum values with all data points included, N=5-18 per group or (B) mean +/- S.E.M. for N=8 per group.

Neuropathic pain mice exhibit anxiety and depressive-like behaviors

Negative affect is a major detriment to quality of life in the chronic pain pathology. We expected our NP pain mice to manifest in negative affective symptoms over time. Thus, we sought to measure two major components of negative affect, anxiety and depression, using the light dark test, forced swim test, and splash test. NP pain mice were inclined to spend less time in the light compartment of the light-dark test (Figure 4.17A), significantly more immobile during the forced swim test (Figure 4.17B), would groom significantly fewer times during the splash test (Figure 4.17C). These behavioral differences demonstrate that NP pain mice have greater negative affect than sham mice. We did not, however, observe any behavioral differences in the open-field test, marble burying test, and the elevated plus maze (data not shown). In general, both sham and NP pain mice explored the center of the open-field arena for approximately similar durations. Even though we did not observe anxiety behavior differences in the open-field paradigm, the locomotor measurements of this test were important because some of the other behavior tests require unhindered locomotion (i.e. forced swim test), which may be confounded by the potential for the CCI procedure to hinder mice mobility. However, no significant differences in mouse locomotor activity between sham and NP pain groups (Figure 4.17D). It has more recently been considered that the marble burying test may be measuring repetitive behavior rather than purely novelty-induced anxiety (Thomas, et al., 2009). Therefore, although we did not observe differences in the number of marbles buried between sham and NP pain animals, they may still be experiencing a form of anxiety that does not influence marble burying. Taken together, these tests demonstrate that mice in NP pain experience negative affect.

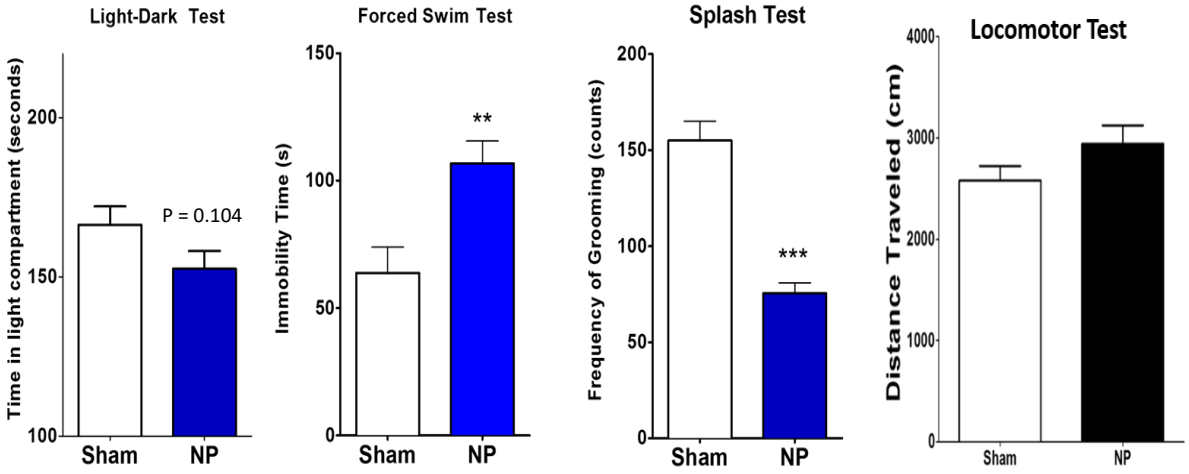

Figure 4.17: A total of 10 sham and 10 NP mice were used for each behavior test. All behavior tests were conducted on the same mice (at different times). (A) Locomotor Test, (B) Light-Dark Test, (C) Forced Swim Test, (D) Splash test. Statistical significance was analyzed via Student's t-test. ** = P<0.01, *** = P<0.001

Kappa opioid receptor inhibition attenuates anxiety and depressive-like behaviors in neuropathic pain

If KOR activation causes the anxiety and depressive-like behaviors associated with chronic NP pain, antagonizing the receptors using the highly KOR-specific JDTic (10 mg/kg) should attenuate these behaviors. Indeed, using a new cohort of 32 mice divided into four treatment groups (sham-saline, sham-JDTic, NP -saline, and NP -JDTic), our results show that pretreatment of JDTic increased time spent in light compartment as well as decreased immobility time for NP pain mice (Figure 4.18). JDTic pretreatment in sham animals did not significantly change their behaviors compared to sham-saline treatment. Furthermore, both males (Figure 4.18A) and females (Figure 4.18B) developed similar levels of anxiety and depressive-like behaviors after NP pain surgery. Overall, these findings clearly demonstrate that KORs are modulating negative affect, and pharmacologically altering solely this receptor's activity levels can influence NP pain mice behavior. Moreover, because both sexes responded similarly to

JDTic pretreatment, blocking KORs systemically to reduce negative affect does not involve sex-specific mechanisms.

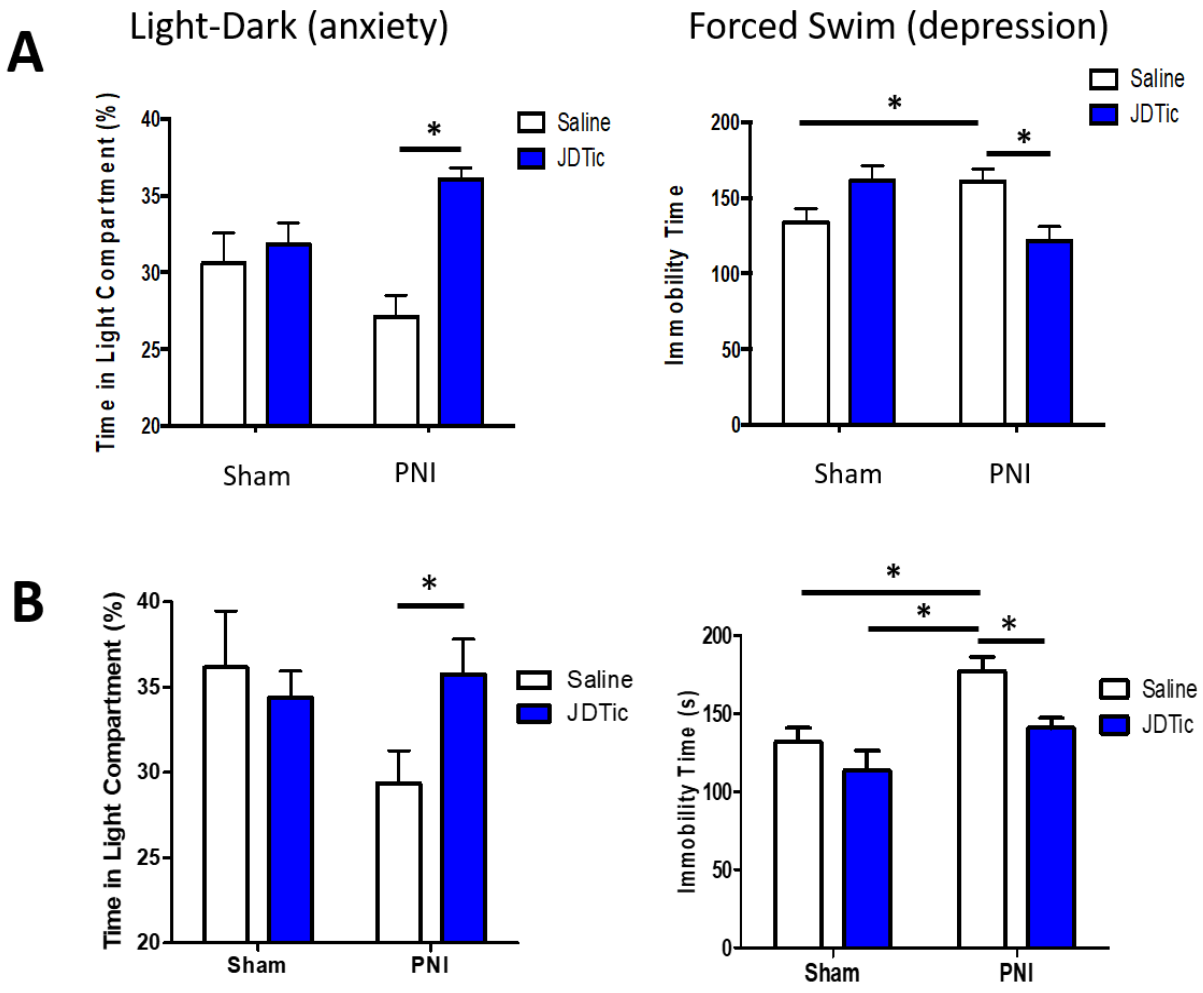

Figure 4.18: NP pain induces anxiety and depressive-like behaviors that are attenuated with KOR antagonist intervention
(A) Light dark anxiety behavior test and forced swim depression behavior on male sham versus PNI mice either given saline or JDTic prior to testing. A total of 8 mice were used per test group. Statistical significance was analyzed via 2-way ANOVA with Bonferroni multiple comparisons. * = $P<0.05$
(B) Light dark anxiety behavior test and forced swim depression behavior on female sham versus PNI mice either given saline or JDTic prior to testing. A total of 8 mice were used per test group. Statistical significance was analyzed via 2-way ANOVA with Bonferroni multiple comparisons. * = $P<0.05$

Discussion

This chapter focused on uncovering the involvement of KORs in chronic pain. While there has been extensive literature on KOR activation in regions that are involved in stress response and reward, this study has further extended our knowledge of the receptor's distribution throughout the brain. In summary, the present study provides the first evidence that chronic pain increases KOR expression and function within the mesolimbic circuitry, which contributes to tonic-aversive component of pain. Considerable evidence suggests that the KOR system within the NAc underlies negative affective states and heightens stress reactivity in various psychiatric disorders. For example, dynorphin expression is increased in the ventral striatum of suicidal individuals and in animal models of depression (Hurd et al., 1997; Peckys and Hurd, 2001). Similarly, the aversive effects produced by KOR agonists are partially mediated by their role in the NAc, where they modulate dopamine transmission from the VTA (Van't Veer et al., 2013). However, no prior study has shown the tonic-aversive component of chronic pain is mediated by KOR; most have proposed no involvement. For example, administration of KOR agonist, but not an antagonist, alleviated acetic acid-induced stretching showing that the KOR agonist alleviated visceral pain, but neither KOR agonist nor antagonist prevented acetic acid-induced place aversion (Bagdas et al., 2016). We have previously shown that KOR agonist-induced antinociception was mediated by engagement of stress pathways, as analgesia could be blocked by pretreatment with anxiolytics (Taylor et al., 2015). Additionally, acute inflammatory or visceral pain was reported to reduce dopamine release in the NAc and depress intracranial self-stimulation (Leitl et al., 2014a; Leitl et al., 2014b); however, neither effect was reversed by pre-treatment with a KOR antagonist, leading to the conclusion that KORs are not involved in pain-induced negative affect. We argue that the KOR system may only be engaged following tissue or

nerve injury that induces a prolonged, chronic pain state but not in pain states that occurs within a short time frame (<72h). Our data supports previous findings using an inflammatory pain model that KOR activation is responsible for the blunted rewarding effects of opioids induced by formalin pain, where KOR antagonists recovered pain-induced attenuation of morphine-reward and evoked dopamine release in the NAc (Narita et al., 2005). However, this study was limited to only exploring the effects of KOR on opioid effects and not on the tonic-aversive component of pain. Since hypo-dopaminergic tone in chronic pain impairs motivated behavior and likely contributes to the occurrence of psychopathology commonly comorbid with chronic pain (Carroll and Carlezon, 2013; Taylor et al., 2015; Taylor et al., 2016), the ability of KOR antagonists to restore dopamine signaling may represent a novel approach to manage such affective dimensions of chronic pain. This mechanism may underlie the effectiveness of buprenorphine (a partial MOR agonist with KOR antagonist properties) in alleviating chronic pain and improving quality of life (Uberall, M. A. and Muller-Schwefe, 2013), and may also explain why buprenorphine when combined with naloxone for treatment of opioid addiction in chronic pain patients also decreased subjective pain (Worley et al., 2015).

CHAPTER 5: DISCUSSION

The role of other glial mechanisms in the brain during chronic pain

In this thesis, we have elucidated important roles of microglia in TBI as well as chronic pain pathology. Aside from microglia, astrocytes are another lineage of glial cells that facilitate and modulate neuronal synaptic communications as well as help repair tissue damage in the brain. They also participate in neuroinflammatory responses such as expressing NFκB (Knoll, Krasnow and Marks, 2017), TNFα and various other cytokines (Banks, Kovac and Morofuji, 2017). Astrocytes are also known to express all three types of opioid receptors in rodents (Ruzicka, et al., 1995). While my studies have not examined astrocytic opioid receptors, it is likely that they may regulate the functions of these cells in the brain after TBI and during chronic pain, especially KORs. Future studies can include examining the role of other cell types such as the macroglia family and how the opioid system is involved in such signaling networks.

Implications of neuroinflammation in traumatic brain injury

The results from this thesis indicate the prevalence of neuroinflammation after rmTBI. In particular, microglia become highly active throughout the brain and beyond just the injury site. Prior research has shown a correlation between neuroinflammatory factors released by microglia (i.e. BDNF) and emotional symptoms after TBI (Wang et al., 2018). We connected microglial involvement to negative affect behaviors such as anxiety and depression in the TBI animals, as pharmacologically inhibiting microglia altered these behaviors. In the literature, sex differences were reported in cases of TBI, such as white matter alterations (Sollman et al., 2017) as well as a more aggressive neuroinflammatory profile in male brains than female (Villapol, Loane, and

Burns, 2017). Our study specifically sought to identify sex differences in our rmTBI model as well. Indeed, TBI produced sex-dependent effects such as female TBI mice developed cold hyperalgesia more significantly than male TBI counterparts. The extent of negative affect measured among the various behavior tests also differed between the sexes. These results support the need to tailor post-TBI treatments to men and women differently in the clinic.

Opioid receptors and traumatic brain injury

Opioid receptors play a critical role in brain plasticity and behavior following TBI. It has been reported that the non-selective opioid antagonist, naloxone, actually exacerbates memory impairment (Morris water maze test) and depressive-like behavior (tail suspension assay) in mice that received mild TBI (Lesniak et al., 2017). Although we did not study the role of opioid receptors in TBI, follow-up studies should include studying opioid receptor mechanisms in this model, especially due to TBI being highly associated with chronic pain. We have connected neuroinflammatory mechanisms of microglia to TBI, and the effects of neuroinflammation in chronic pain states, as well as the involvement of KORs that lead to mood disorders in rodents. It is highly possible that KORs are also implicated in neuroplasticity following TBI. One study reported that KOR agonist RU599 had a neuroprotective and analgesic effect against TBI (Ikeda and Matsumoto, 2001). Since this report, follow-up studies on this receptor's activity and the behavioral deficits in TBI animals have been minimal. However, studying the KOR system in the TBI model may be a promising avenue to identify novel therapeutics for treating patients with negative symptoms resulting from TBI.

Neurocircuitry of affect in chronic pain

Currently, pain research has shifted away from conventional opioid treatments (involving targeting the MOR) and expanded towards alternative endogenous opioid targets. KORs are still part of the opioid receptor family and share the analgesic property when activated. The drawback of fully activating *any* opioid receptor is the potential side effects that occur. With MOR agonists, side effects may include constipation due to MOR presence in the intestinal tract that lead to peristaltic inhibition (Chen, Chung, and Cheng, 2012), respiratory depression, slight blood pressure reduction, or possible sedation. These problems can severely limit their use. Likewise, KOR agonists pose severe side effects as well, which stem from the alternate pathways of action involving β-arrestin, MAPK, and JNK signaling (Figure 5.1). Due to these challenges, more studies on KOR pharmacology have led to biased agonism (focused on the analgesic pathway and preventing the alternate pathways) as a promising option for KOR-based pain therapy. Some possible biased KOR agonists have seen positive results in the clinic as well.

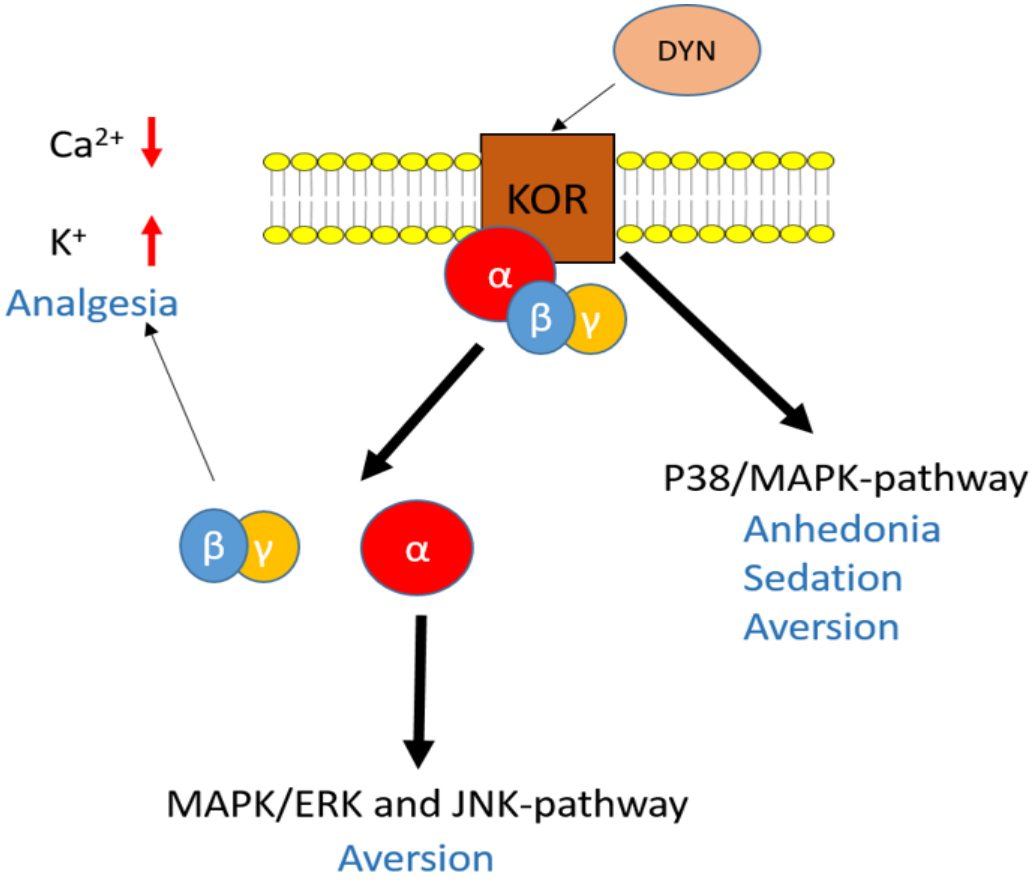

Figure 5.1: Diagram of KOR intracellular signaling. KOR GPCR subunits dissociate upon binding to a ligand (in this case dynorphin, Dyn). The subunits beta-gamma lead to changes in ion concentration that depolarize the cell and prevent pain signaling (analgesia). The alpha subunit may lead to downstream kinase activation and transcriptional effects that could signal more long-term side effects (aversion). KOR also undergoes desensitization and arrestin signaling that leads to p38/MAPK secondary pathways and trigger anhedonia.

Although biased KOR agonism is a potential pain therapeutic at the receptor level, it is extremely important to be aware of the overall changes that KORs have in the brain during *chronic* pain. Our understanding of pain signaling at the brain level has resulted in many ways to treat acute pain; however, effective chronic pain treatments still elude us. The effectiveness of long-term pain management diminishes over time because chronic pain involves many complex neurochemical and circuit-level changes in the brain. Therefore, my dissertation provides additional insight to how these changes occur. KORs are expressed in the reward and stress pathways (Figure 5.2A) and become upregulated in these brain regions when chronic pain occurs (Figure 5.2B). KOR activation suppresses dopamine signaling throughout these pathways and leads to negative affect. Although this study characterizes a significant amount of KOR signaling mechanisms, some further details of this receptor's involvement during pain still need to be elucidated. For example, this study found that KOR expression and activity are increased in the NAc during chronic pain. Through gene expression analysis, it was also observed that KORs are likely expressed on prodynorphin-expressing GABAergic MSNs at the NAc. In addition, it is suggested that KORs expressed at the VTA dopamine neurons are transported to the terminals that reach the NAc (Mansour, Fox, Akil, and Watson, 1995; Britt and McGehee, 2008). However, this study does not conclusively distinguish which of these cell types contribute to the KOR upregulation in the NAc. It could be possible that KORs in either the GABAergic MSNs or dopamine terminals are upregulated, or even both. Our study also showed increased KOR activity in all sub-regions (medial, central and basolateral) of the amygdala. However, these regions are innervated by multiple distinct neural projections from other regions, and more research needs to be done to fully elucidate the contribution of KOR activity within amygdala

sub-regions during chronic pain. Furthermore, we have observed different populations of prodynorphin and proenkephalin cells in these sub-regions, and different levels of KOR gene expression colocalized to these cell populations. Although we noted these neurobiochemical changes in the chronic pain state, we do not know specifically which of these regional KOR increases contribute the most to the anxiety-like behavioral outputs of our chronic pain animals. Finally, the VTA is a highly heterogeneous mix of various cell types other than dopamine neurons. GABAergic neurons play critical role in regulating dopamine signaling, and the topology of the VTA is still under active investigation in the neuroscience field. This study has uncovered potential KOR expression on GABAergic neurons at the VTA, and not exclusively on dopamine neurons. Moreover, in chronic pain, it is possible that KORs are upregulated in GABA neurons at the VTA as well. I hypothesize that these KORs are primarily in the interneurons within the VTA that may disinhibit direct GABA inputs to the dopamine neurons, ultimately generating another form of dopamine signal suppression at the VTA level and not only at the NAc level. This prediction has not yet been validated through our study and needs follow-up experimentation to confirm. Overall, while there is still much to learn about KOR signaling mechanisms, it is evident that they perform important roles in in the brain during chronic pain, and the results of this dissertation support the idea that suppressing KOR activity in at least some of these mechanisms could be effective therapeutic options for treating the *mood components* surrounding pain, and not merely on the popular *analgesic* therapeutic directions.

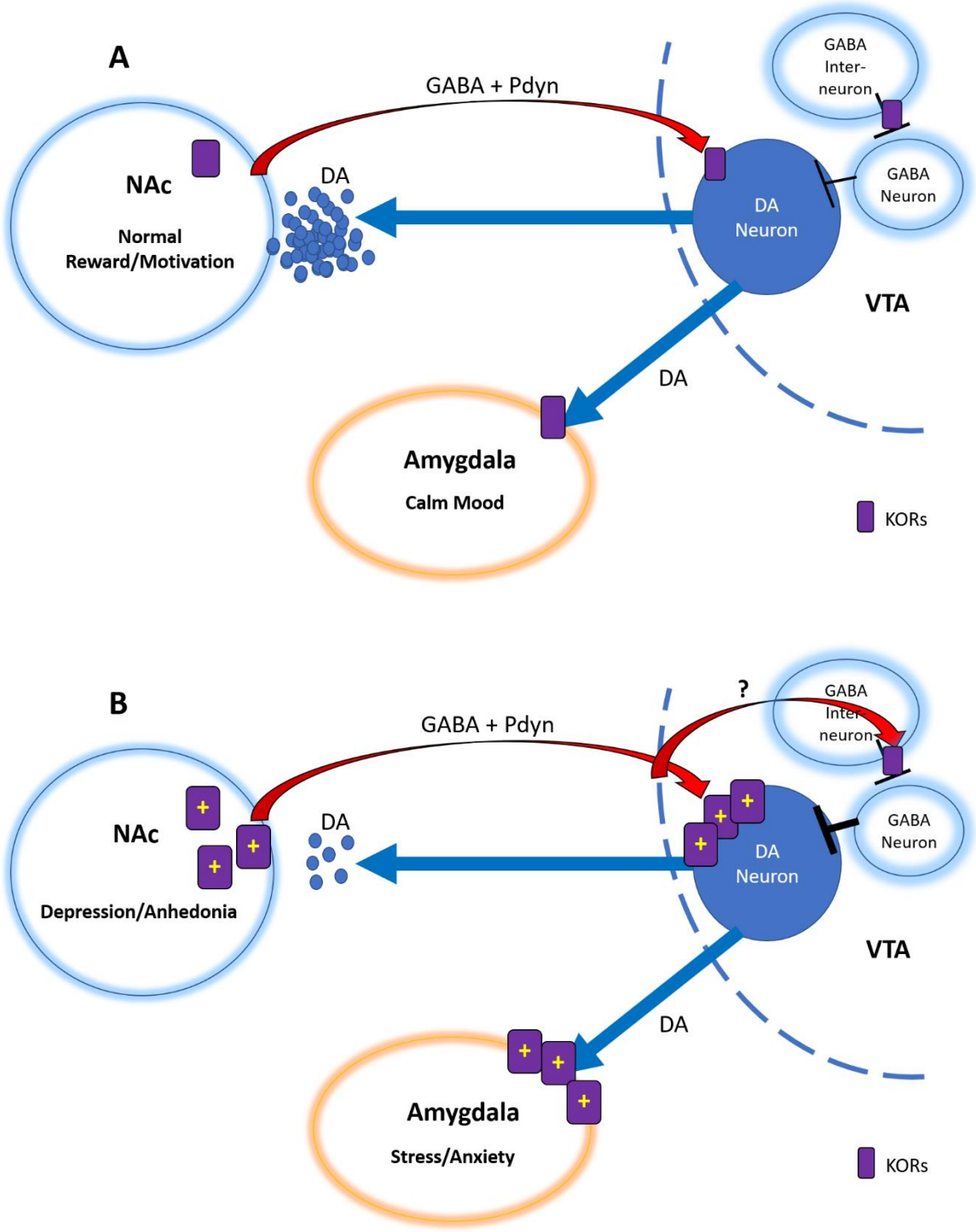

Figure 5.2: Neurocircuitry in the normal state **(a)** and the chronic pain state **(b)**. Dopamine signaling is blunted in chronic pain. KORs are upregulated at the NAc, amygdala, and VTA during chronic pain that results in mood disorders such as depression and anxiety.

Kappa opioid receptors and neuroinflammation

Based on the results described in Chapter 2, microglia in the brain are activated in response to NP pain. Chapter 4 showed that KORs in the brain are highly upregulated during NP pain as well. It may be possible that these responses also regulate each other. In general, opioids have been known to exert anti-inflammatory effects in the brain and the periphery. They can block NFκB signaling in T cells (Borner and Kraus, 2013). In a model of brain damage inflammatory response via ischemia and reperfusion, it was shown that KOR activation inhibited inflammation (Chen et al., 2016). In NP pain, it is possible to postulate that neuroinflammation mediated by microglia activation is important for modulating the processing of the pain. However, since the pain is chronic, this inflammatory response becomes maladaptive, and KOR upregulation may be the brain's compensatory mechanism to control it. Yet, this heightened KOR activation could also produce maladaptive effects that exacerbate pain in the long term. Overall, the mechanisms, or even the possibility, that KORs regulate microglia activity during chronic pain are not currently well-studied but should be examined in follow-up studies.

Potential microglial interactions on dynorphin and kappa opioid receptor signaling

The studies outlined here have provided us more understanding of both microglial and KOR involvement in chronic pain conditions. The next challenge is to ascertain whether microglia or KORs regulate and facilitate each other's activation during pain. Prior evidence in the literature suggests that pruritis through KOR blockade at the periphery leads to scratching; the scratching can induce microglial activation at the spinal cord level (Zhang et al., 2015). The activation of microglia in this scenario could be prevented using a the KOR-selective biased agonist, nalfurafine. Another study has shown that KOR activation promotes microglial

proliferation in the central nervous system in a model of trigeminal neuropathic pain (Aita et al., 2010). This coincides with other research conducted on the KOR ligand, dynorphin, which has been shown to increase macrophage superoxide production (Sharp et al., 1985), modulate their oxidative (Tosk et al., 1993) and phagocytotic activity (Foster and Moore, 1987), and increase their proinflammatory cytokine signaling (Apte, Durum and Oppenheim, 1990). It has also been shown that BDNF signaling on TrkB receptors causes downstream MAPK activation and a subsequent upregulation of dynorphin, which leads to increased KOR activation (Logrip, Janak and Ron, 2008). Although this study was conducted in and ethanol consumption model, we can extrapolate similarities in our PNI animal model, whereby PNI induces microglial release of BDNF in the brain and potentially downstream KOR activation. In our study of KORs and microglia in PNI mice, we conducted bioinformatics analysis using promoter-transcription factor interaction tools to determine whether BDNF directly leads to increased KOR transcription through possible promoter enhancement upstream of the KOR gene. We found a potential interaction between BDNF and the upstream promoter site, Sp1, approximately 784 base pairs away from the KOR gene. Although these are only predictive models, there is a possibility that neuroinflammation and KOR upregulation are joint mechanisms that facilitate each other to produce the vast negative consequences observed in NP pain.

Chronic pain and kappa opioid receptors in regulating other neurotransmitters

We have demonstrated that KORs are activated in chronic pain states, causing a dysfunction of dopamine signaling that may be responsible for the various behavioral deficits in our rodent models. Although my thesis has thoroughly studied the dopamine signaling in these models, other neurotransmitter signaling pathways exist in the brain that remains to be explored.

One example would be the serotonin signaling network. Since KOR activation in response to chronic pain leads to negative affect such as depression, it is possible that these receptors are also interfering with serotonin release or reuptake in the brain. A recent study demonstrated that KOR activated decreased serotonin uptake through altering the neurotransmitter's trafficking via phosphorylation mechanisms (Sundaramurthy et al., 2017). I predict that this phenomenon may be occurring in chronic pain as well, which could be another mechanism contributing to pain-associated negative affect. Many types of antidepressants on the market involve modulating serotonin release, and recent studies have shown that KOR antagonists can perform those roles as well (Li et al., 2016). Recent studies involving KOR signaling and behavior tend to focus on dopamine-driven circuits and intentionally filter out any involvement of serotonin signaling pathways (Abraham et al., 2017), but serotonergic cells have also been shown to control the affective component of pain (Singh et al., 2017). Moreover, KOR agonist-induced place aversion requires dorsal raphe nucleus serotonergic system control (Land et al., 2009). This alludes to the possibility that chronic pain aversion through KOR upregulation evidenced in my studies could also be mediated by the serotonergic system as well. Although my studies have shown no significant KOR expression upregulation in cells within the raphe nuclei of NP pain animals, other downstream projections from the raphe could be investigated further. KOR agonist-stimulated GTPγS binding results depicted previously in this manuscript have shown that the cingulate cortex, preoptic areas and hypothalamic nuclei, which all receive input and in turn feedback to the raphe nuclei, have abundant KOR activity. Future directions may include studying the role of KORs in these regions and behavioral deficits in the chronic pain model.

Age-dependent effects on kappa opioid receptor activation in chronic pain

Age is an important factor in assessing prevalence and severity of chronic pain in humans. Some types of chronic pain tend to manifest later in life than others. In addition, age can alter sensory pathways, leading to changes in how one is able to adapt to the pain. It has been acknowledged that KOR constitutive activity occurs in the PFC of young rats; but such activity declines with age (Sirohi & Walker, 2015). KOR activation response at the CPu also varies depending on age in evoked dopamine microdialysis studies (Cortez et al., 2010). Future studies in this topic may involve using adult mice that are 24 weeks (6 months old) undergoing either NP pain or TBI, as well as comparing pain pathologies to the results obtained from mice in the current study.

Involvement of kappa opioid receptor signaling in non-pain related anxiety and depression

KORs have been known to regulate anxiety and depression phenotypes even in non-chronic pain conditions. It has been observed that mutations or epigenetic modifications to the promotor of KOR can trigger dysfunctional KOR expression that is highly associated with alcohol addiction and even suicidality (Lutz et al., 2018). Either stress or KOR-selective agonists can generate increased anxiety in non-chronic pain rodents within the elevated plus maze paradigm; these anxiety behaviors could be attenuated with a KOR antagonist (Peters et al., 2011; Valdez and Harshberger, 2012). These mechanisms are also thought to involve the endogenous ligand, dynorphin, and its downstream regulation of corticotrophin-releasing factor (Van't Veer and Carlezon, 2013). Furthermore, short-acting antagonists for KORs such as LY2456302 are being developed in an effort to treat depression (Urbano et al., 2014). Some of the anxiety and depressive-like behaviors triggered by increased KOR tone in the brain may also be key factors leading to withdrawal states of drug addiction (Butelman, Yuferov and Kreek,

2012). Thus, pharmacologically blocking KORs can decrease the depression phenotype in cocaine withdrawal rodents (Chartoff et al., 2012). Overall, the role of this receptor in modulating mood is present not only in our studies on chronic pain, but also in general anxiety disorders and depression. Based on the insights gained from studying KOR function in chronic pain, we can use this understanding to treat mood disorders in non-pain related conditions as well.

Closing Remarks

By understanding the neurobiological mechanisms of the somatic and affective components of pain, we can address the growing epidemic of opioid abuse in society. In the past two decades, there has been an approximately 500% increase in opioid-related deaths in the United States (Dowell, Noonan and Houry, 2017). A significant contributor to these drug-related overdoses is improper pain management and over-prescribing of opioid painkillers. As we learn more about how pain is processed, we can find alternative non-addicting therapeutics that may also be less likely to develop drug tolerance. Together, such new treatments could drastically reduce the amount of potential drug abuse.

Pain is often underestimated in terms of its pervasiveness and impact it has on the human population. When we discuss health problems in the world, some common topics that arise typically include cancer, heart disease, and age-related illnesses. Pain is *not* the first topic that comes to mind. However, the common trait among almost all of these diseases is pain. This issue pervades a multitude of health problems and significantly debilitates the patients who are struggling to cope with diseases. In conclusion, understanding the mechanisms of pain is crucial

for the development of novel therapeutics that will ultimately improve patient quality of life across a wide range of illnesses, injuries, and diseases.

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