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SPINAL KAPPA OPIOID RECEPTOR ACTIVITY INHIBITS ADENYLYL CYCLASE-1 DEPENDENT MECHANISMS OF CHRONIC POSTOPERATIVE PAIN

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Digital Object Identifier: https://doi.org/10.13023/etd.2019.211

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SPINAL KAPPA OPIOID RECEPTOR ACTIVITY INHIBITS ADENYLYL CYCLASE-1 DEPENDENT MECHANISMS OF CHRONIC POSTOPERATIVE PAIN

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By
Lilian Custodio
Lexington, Kentucky

Director: Dr. Bradley K. Taylor, Professor of Physiology

Lexington, Kentucky
2019

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ABSTRACT OF DISSERTATION

SPINAL KAPPA OPIOID RECEPTOR ACTIVITY INHIBITS ADENYLYL CYCLASE-1 DEPENDENT MECHANISMS OF CHRONIC POSTOPERATIVE PAIN

Chronic postoperative pain impacts millions of individuals worldwide that undergo a variety of surgical procedures. Opioids remain the mainstay analgesics of acute and perioperative pain; however, prolonged opioid therapy may lead to life-threating adverse effects, tolerance, dependence, and addiction. Therefore, unraveling the cellular mechanisms that drive persistent pain states and opposing endogenous analgesia provided by opioid receptor signaling, may lead to novel analgesics. Evidence suggests that tissue injury leads to increased sensitization of the spinal cord nociceptive neurons which increases susceptibility to chronic pain via an N-methyl-D-aspartate (NMDA) receptor activation of calcium-sensitive adenylyl cyclase isoform 1 (AC1). This phenomenon, named latent pain sensitization (LS), is mediated by a compensatory response of endogenous inhibitory systems.

In this dissertation, we test the hypothesis that surgical insult promotes prolonged activation of kappa opioid receptors (KOR) which mask LS via attenuation of pro-nociceptive AC1 signaling pathways in both male and female animals. We employed a murine model of chronic postoperative pain that promotes LS in the spinal cord and closely resembles the phenotypic features of postoperative pain in human subjects. When behavioral signs of hyperalgesia resolved, we targeted spinal opioid receptor systems and pronociceptive modulators with intrathecal delivery of selective pharmacological antagonists and assessed behavioral signs of hyperalgesia and spinal nociceptive sensitization. We propose that LS is kept in remission by a long-lasting compensatory response of tonic endogenous KOR signaling that hinders a pronociceptive LS pathway that includes not only AC1 but also two downstream targets: protein kinase A (PKA) and exchange protein activated by cAMP (Epac1/2) - in a sex-dependent manner. Our results propose new therapeutic targets for the management of persistent postoperative pain and underscore the importance of tailoring sex-specific pain management strategies.

KEYWORDS: Postoperative pain, latent pain sensitization, kappa opioid receptor, adenylyl cyclase -1, protein kinase A, exchange protein activated by cAMP.

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04/26/2019

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SPINAL KAPPA OPIOID RECEPTOR ACTIVITY INHIBITS ADENYLYL CYCLASE-1 DEPENDENT MECHANISMS OF CHRONIC POSTOPERATIVE PAIN

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ACKNOWLEDGMENTS

First, I would like to acknowledge my advisor, Dr. Brad Taylor for the opportunity of working in his laboratory and mentoring me even after relocating to Pittsburgh and enabling me to complete this work and continue my journey for knowledge. I am also grateful to the following individuals, among others, who contributed and assisted with my graduate training and completion of this project. Dr. Bret Smith for his constant encouragement and valuable guidance in this project. To my friend, Dr. Romulo Albuquerque who served as the PI of the animal protocol in the last year of this project. He is one of the outstanding professionals who inspired me to pursue this career trajectory. Dr. Liping Zhang for her ability to assist me and bring levity to my spirits at times when the stress was great. Renee Donahue for showing up in the laboratory at all times to support me, help me with techniques and for her friendship.

I wish to thank my committee members: Drs. Bret Smith, Gregory Frolenkov and Reny de Leeuw for their mentorship and constructive criticism during my graduate training. I thank Dr. Eric Blalock for serving as the outside examiner for my dissertation defense. I also would like to express my deep gratitude and appreciation for Dr. Linda Dwoskin and the NIDAT32016176 faculty for their mentorship, financial support and valuable training in the field of substance use disorders. Moreover, I acknowledge all the faculty of the department of Physiology and the staff Allison Walters, Andrew Hernandez, Tanya Graff for their support, and Tanya Seward for managing our animal colony.

Finally, I would like to thank my family while separate by a great distance have always supported my endeavors. Especially my father Afranio who taught me that integrity, diligence and extreme perseverance are fundamental keys to achieve your goals, even when they take an unexpected shift. For my supportive and loving husband Edward and lovely son Andrew who provided on-going support and motivation despite my constant absence throughout this process.

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CHAPTER 1. Introduction and Overall Hypothesis

1.1 Introduction

Chronic pain is an emergent cause of morbidity and long-term disability worldwide. With an estimated minimum 1/3 of the adult United States population suffering from chronic pain, the costs in healthcare account for approximately $600 billion annually (Medicine, 2011). In fact, 11.2% of Americans report moderate to severe daily pain (Nahin, 2015), and 4.8% of chronic pain patients exhibit high rates of disability, psychological and cognitive impairment (Pitcher et al., 2018). The increased burden of chronic pain is exacerbated by the fact that many patients are refractory to available pharmacological interventions. For instance, almost 20% of patients with neuropathic pain are placed on long-term opioid regimens as the ultimate resource to manage the pain severity, improve their quality of life and functional impairment (Eriksen et al., 2006; Hoffman et al., 2017). Among the chronic pain patients on prolonged opioid therapy, over 60% report having initiated opioids following surgical procedures (Callinan et al., 2017). Long-term opioid therapy, however, it is only moderately effective *at best* and can lead to not only life-threatening adverse effects, but also analgesic tolerance, dependence, abuse liability and paradoxical pain exacerbation (Eriksen et al., 2006). Therefore, there is an increased need for multidisciplinary approaches and research leading to novel and efficacious non-opioid treatment modalities for chronic pain.

One of the significant challenges in pain research is to understand the mechanisms of chronic pain and its inhibition. There is a consensus that persistent pain states result from maladaptive plasticity of a network of neurons in the CNS, including the dorsal horn

of the spinal cord (Sessle, 2000; Latremoliere and Woolf, 2009; Sandkuhler and Gruber-Schoffnegger, 2012). Tissue injury or inflammation produce increased excitability of nociceptive circuits that are driven by N-methyl-D-aspartate (NMDA) glutamatergic receptor dependent activities in the central synapses and their downstream pathways (Woolf, 2007; Zhuo, 2012). Thus, uncovering the cellular mechanisms that are involved in pain-related plasticity is critical for developing more effective pharmacological approaches.

It is well recognized that spinal nociceptive transmission is modulated by activation of opioid receptors in the peripheral and central nervous system (CNS) upon coupling of exogenous agonists or its endogenous ligands (Yaksh, 1987). We postulate that tissue injury is followed by a period of long-lasting pain vulnerability entitled latent sensitization (LS), in which sensitized spinal nociceptive pathways remain quiescent due to compensatory activity of pain inhibitory systems in the dorsal horn (Solway et al., 2011; Corder et al., 2013) that outlasts the resolution of the injury. Disruption of the inhibitory systems due to subsequent exposure to an injury (Taylor and Corder, 2014; Pereira et al., 2015b) or stressful events (Rivat et al., 2007; Marvizon et al., 2015) can trigger episodes of hyperalgesia. The silent phase of LS can be unmasked upon administration of the non-selective antagonist/inverse agonist of opioid receptors-naltrexone or naloxone in rodents (Campillo et al., 2011; Corder et al., 2013), and in a subpopulation of susceptible human subjects (Pereira et al., 2015b). This phenomenon indicates a compensatory endogenous opioid mechanism after injury, where mu opioid receptors (MOR) become constitutively active (MOR_{CA}) to combat opposing processes of pain transduction, thereby possibly preventing the transition from acute to persistent pain states. However, the contribution of

other opioid receptor subtypes to LS remains to be determined. Thus, it is conceivable that kappa opioid receptors (KOR) may also exert a compensatory long-lasting antinociceptive response to repress hyperalgesia after injury.

MOR_{CA} suppresses inflammatory pain in part, via inhibition of AC1 - cAMP signaling during LS. Thus, pharmacological blockade of MOR_{CA} induces pain reinstatement possibly through disinhibition of NMDA-AC1 pathway allowing an increase in cAMP and downstream signaling transduction (Corder et al., 2013; Taylor and Corder, 2014). Recent evidence indicates a role of the c-AMP downstream target protein kinase A (PKA) and exchange protein associated with cAMP (Epac) in LS (Fu et al., 2019). Less appreciated; however, is whether males and females differ in LS endogenous control and spinal maladaptive plasticity mechanisms. Therefore, we propose that endogenous postoperative analgesia would be differentially mediated by tonic KOR-mediated inhibition of AC1 and/or downstream c-AMP signaling proteins PKA and Epac in male and female mice.

1.2 Overall Hypothesis and Specific Aims

This dissertation tests the overall hypothesis that tissue injury leads to sustained activation of kappa opioid receptors (KOR) in the dorsal horn of the spinal cord that maintains latent sensitization (LS) within a state of remission, via inhibition of adenylyl cyclase-1 (AC1)-dependent pathways in male and female mice. To test this hypothesis, I designed and performed several experiments to examine the following specific aims:

1. Delineate the contribution of the opioid receptor subtypes to the remission phase of LS.

2. Determine whether there are sex differences in postoperative endogenous opioid receptor analgesia.
3. Examine whether KOR antagonist-induced reinstatement of hyperalgesia is associated with spinal nociceptive neuronal sensitization.
4. Determine whether KOR postoperative endogenous analgesia (suppression of LS) is mediated by inhibition of AC1 and/or downstream PKA and Epac mechanisms.
5. Investigate potential sex differences in KOR-dependent LS.

CHAPTER 2. General Background

2.1 Overview of Pain: concepts and classification

Pain is a complex multidimensional experience. Subjective in nature, and intrinsically associated with emotional and cognitive factors, it is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey H & Bogduk N, 1994). Thus, the pain experience encompasses both a sensory-discriminative and affective-motivational component. The first component denotes the perceptual nature of pain; location, intensity, and quality of the noxious stimuli can be distinguished via neural processing in the thalamus and somatosensory cortices. While, the latter refers to its psychological aspects mediated by the limbic system (Basbaum et al., 2009; Braz et al., 2014). Both pain dimensions can be assessed experimentally. The sensory component is commonly measured in rodents and human laboratory studies using stimulus-evoked tests such as von Frey filaments (Tena et al., 2012; Wildgaard et al., 2012), and thermal assays (Woolfe, 1944; Hargreaves et al., 1988). These assays employ physiological stimuli (i.e., mechanical, thermal, chemical) to the limbs (or face) to assess changes in pain thresholds determined by the withdrawal or escape latency from innocuous or noxious stimuli (Deuis et al., 2017). Similarly, the affective-motivational dimension of pain is characterized by a negatively reinforcing component that can be defined in preclinical studies using motivation to seek pain relief assays in rodents such as Conditioned Place Preference or Conditioning Place Avoidance paradigms (He et al., 2012; Griggs et al., 2015). While, it is assessed in the clinical setting by self-report pain scales or self-administered questionnaires (Hardt et al., 2000; Martin et

al., 2019). This dissertation will focus primarily on stimulus-evoked tests (von Frey-filament) to assess mechanical hypersensitivity following surgery and pharmacological manipulations of the opioid receptor systems.

As previously highlighted, pain is highly subjective and relies on patients' self-report. Thus, the term nociception, defined as neural processes of noxious stimuli detection and processing (Basbaum et al., 2009) better applies to preclinical research, thereby it will be used throughout this work. Acute (nociceptive) pain is fundamental for survival, as it serves as a protective apparatus to inform the organism about imminent threats to avoid further tissue damage and allow healing. Nociceptive stimuli are encoded by specialized subpopulations of peripheral neurons, so-called nociceptors that transmit the noxious signals to the first relay center of information in the CNS, the dorsal horn of the spinal cord (or subnucleus caudalis in the brainstem for facial and cephalic stimuli). There the noxious signals are processed and modulated before ascending to the higher centers in the brain where the pain is perceived. The importance of acute pain is underlined in individuals with congenital insensitivity to pain, in which the inability of detecting noxious stimuli due to non-functional nociceptors leads to repeated severe injuries and reduced life expectancy (Cox et al., 2006; Goldberg et al., 2007).

Pain is often categorized based on its duration. Acute pain is short-lived, lasting from minutes to several weeks, and has a distinct etiology, such as tissue damage, inflammatory processes or surgical intervention. If undertreated, acute pain may progress to persistent pain states, lacking its biological function and becoming a disorder itself. Chronic pain is multifaceted, accompanied by physical and psychiatric comorbidities, and is, therefore, one of the most critical challenges in the clinic setting. It can exacerbate over

time and persist from 4 months (IASP) to years, or even a lifetime. It may arise from unremitted injuries (low back pain, postoperative pain), or be associated with a disease (i.e., rheumatoid arthritis, diabetes). However, many of these clinical entities lack a clear etiologic factor, such as fibromyalgia and chronic daily migraines. In an attempt to solve this gap in the pain classification, the IASP recently adopted the terminology of nociplastic pain to embrace the conditions that arise from altered nociception but are devoid of a recognizable causal factor or associated disease (IASP, 2017). Moreover, chronic pain patients can suffer from constant pain or experience episodes of remission as in low back pain or persistent postoperative pain. In this dissertation, we adopted a preclinical model of persistent postoperative pain due to its high prevalence and incidence, and identifiable etiology that allows the investigation of putative mechanisms involved in the transition from acute to chronic pain.

2.2 Postoperative pain: epidemiology and therapeutics

It is estimated that approximately 40% of chronic pain patients report that their symptoms were initiated after trauma or surgical intervention (Schug SA & Pogatzki-Zahn EM, 2011). Over 300 million surgeries are performed annually worldwide (Weiser et al., 2016) and up to 80% of patients may experience postsurgical pain (Apfelbaum et al., 2003). Among them, women report higher postoperative pain levels than men (Storesund et al., 2016), and recent evidence indicates that females are at higher risk to develop persistent pain after surgery (Schug AS & Pogatzki-Zhan EM, 2011). Persistent postoperative pain is a significant burden to society as it impairs the quality of life of 5% to 85% of patients that undergo a variety of surgical procedures every year (Schug AS & Pogatzki-Zhan EM, 2011). It is defined as constant or intermittent pain in the surgical site (primary

hyperalgesia) or surrounding area (secondary hyperalgesia) that persists for at least three months after the surgical procedure (Macrae, 2008; Werner and Kongsgaard, 2014). The pain exhibits both inflammatory and neuropathic features as transoperative nerve damage may occur (Kehlet et al., 2006; Haroutiunian et al., 2013).

The increased rate of chronic postoperative pain reflects, at least in part, the inadequacy of currently available treatments. It is also possibly due to a disconnect between the findings of fundamental research and clinical practice (Gerbershagen et al., 2014; Cooper et al., 2016). To date, opioids remain the first line of treatment for acute postoperative pain; however, its adverse effects including nausea, vomiting, and constipation, the risk of respiratory depression, dependence, and addictive properties compromise its effectiveness (Al-Hasani and Bruchas, 2011). Moreover, opioids are less efficacious in cases with a strong neuropathic component, and when used preoperatively increase the likelihood of persistent pain after surgery (VanDenKerkhof et al., 2012). Current guidelines advise multimodal protocols encompassing cyclooxygenase inhibitors-2 (COX-2) and NMDA antagonists. When neuropathy is present, drugs that reduce neurotransmission and membrane stabilizers are recommended perioperatively (Chou, 2016). A recent meta-analysis supports the use of the α2δ ligands, gabapentin and pregabalin, perioperatively to reduce the risk of persistent surgical pain (Clarke et al., 2012). Despite the multitude of available therapies to treat acute postoperative pain, there is no highly effective prophylactic drug to manage chronic postsurgical pain. Therefore, drugs with potent analgesic properties and low addictive effects are warranted.

Here, we adopted a preclinical model of postoperative pain where a 5mm incision is performed on the mouse hindpaw through the glabrous skin, fascia and plantaris muscle

under general anesthesia, precipitating non-evoked guarding behavior of the limb that lasts 1 to 2 days and mechanical hypersensitivity that persists up to 3 weeks (Pogatzki and Raja, 2003). The intraoperative procedure of skin and fascia incision, muscle retraction and behavioral presentation closely recapitulate the postoperative pain phenotype in patients (Pogatzki-Zahn et al., 2007; Pogatzki-Zahn et al., 2017). After hyperalgesia subsides, pharmacological approaches are used to target the opioid receptor system and key pain modulators of spinal neuroplasticity, which in turn leads to reinstatement of pain behavior and replicates persistent postsurgical states in patients. This model allows us to investigate potential novel therapeutic targets involved in chronic pain consolidation such as AC1 and downstream proteins Epac1/2.

2.3 Anatomy of nociceptive circuitry in the dorsal horn of the spinal cord

In the periphery, free nerve terminals from primary afferent neurons transduce noxious stimuli into electrical signals (action potentials) that are conveyed to the CNS. The dorsal horn of the spinal cord is the primary point of conversion of nociceptive inputs from the periphery to high centers in the brain. The dorsal horn receives information from heterogeneous groups of neurons responsible for processing and intense modulation of noxious inputs before these signals ascend to the brain, where pain perception occurs. The dorsal horn neurons are comprised of four groups: (1) central terminals of primary afferents; (2) interneurons; (3) projection neurons; (4) descending axons from the brainstem (Todd A and Koeber H, 2015). This work focuses on the first three populations of neurons involved in the processing and modulation of nociceptive signals in the spinal cord.

The dorsal horn neurons are organized within a distinct laminar pattern (I-VI) according to their functional class (Rexed, 1952). The superficial dorsal horn is the primary region of relay for the primary afferent neurons and is comprised of laminae I or marginal layer, and lamina II or substantia gelatinosa. Interneurons predominate in laminae I-III, while the deeper laminae IV-VI are sites of synapse of primary sensory and projection neurons **(Figure 2.1.A)** (Basbaum et al., 2009; Braz et al., 2014; Todd, 2017).

2.3.1 Primary sensory afferent inputs into the dorsal horn

Nociceptive primary afferents are specialized peripheral sensory neurons that encode intense, noxious (i.e., mechanical, thermal, chemical) stimuli from the periphery into the dorsal horn. Nociceptors are pseudo-unipolar neurons; the body of the neuron arising from the limbs and trunk lies on the dorsal root ganglia (DRG). Their axons diverge from the DRG to give rise to peripheral branches that innervate the skin, viscera, musculoskeletal system, and blood vessels; and central terminals that synapse with second-order neurons in the dorsal horn.

The primary afferent neurons are somatotopically organized in humans and animals. In mice, the innervation of the hindpaw and lower limbs is supplied by three subdivisions of the sciatic nerve: tibial, common peroneal and sural nerves that emerge from the lumbar spinal nerves L3-L5. The sciatic nerve in mice is composed predominantly of fibers originating at the L3 and L4 DRGs. Retrograde labeling and behavioral studies demonstrated that in the C57Bl/6 mouse strain, the primary anatomical and somatosensory contribution is predominantly provided by L4 (Rigaud et al., 2008). Therefore, in our immunofluorescent assay, we evaluated the cellular markers at the L4 segment of the dorsal horn of the spinal cord.

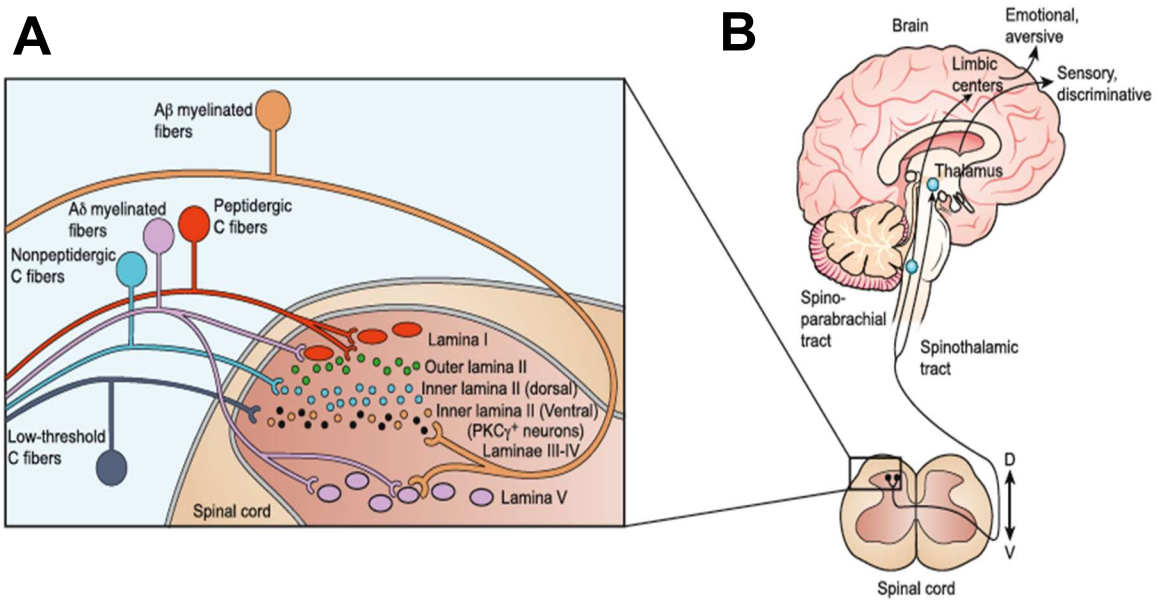

Figure 2.1. Dorsal horn pain transmission pathways.

(**A**) Close sagittal view of the dorsal horn of the spinal cord. The distinct populations of primary afferent fibers synapse with projection neurons in laminae I (red) and V (purple), and interneurons from laminae II outer (green) to IV (black). (**B**) Ascending tracts from the dorsal horn to the higher centers of the brain [Adapted from Braz et al., 2014].

Based on their physical properties, the nociceptors are classified into two main groups: A-delta (Aδ), and C-fibers. Aδ fibers are medium in diameter and thinly myelinated, with conduction velocity >2m/s. They project to lamina I and V where they synapse with projection neurons. They are highly discriminative and are thought to mediate fast, sharp, aching, well-localized pain. Conversely, C-fibers are small in diameter, unmyelinated, and therefore have slower conduction velocity (< 2m/s). The C-fibers are subdivided into peptidergic and non-peptidergic subsets, expressing substance P (SP), calcitonin gene-related peptide (CGRP), and transient vanilloid receptor, isoform 1 (TRPV1) receptors; and isolectin IB4 respectively. The peptidergic C-fibers project to lamina I and lamina II outer; whereas non-peptidergic fibers project to lamina II inner and synapse with interneurons, creating complex circuits within the dorsal horn. C-fibers possibly mediate more diffuse and slow pain. Both, C- and Aδ fibers are polymodal, responding to tactile, thermal and chemical stimuli.

The third subtype of peripheral sensory afferents is the large diameter and fast conduction A-beta (Aβ) fibers. They project to lamina III-V and are responsive for low threshold mechanical stimuli in physiological conditions **(Figure 2.1.A)**. In the injury setting, they become responsive to noxious stimuli, leading to increased hypersensitivity to innocuous tactile stimulation, named allodynia (Basbaum et al., 2009; Todd, 2010; Braz et al., 2014).

2.3.2 Dorsal horn interneurons

The nociceptive inputs delivered by the primary sensory neurons in the dorsal horn are the subject of intense modulation by interneurons. Interneurons outweigh the other neuronal cells throughout the dorsal horn. For example, the superficial lamina is composed

of a small fraction (> 5%) of projections neurons (Campbell et al., 1998), and at least 90-95% of interneurons (Spike et al., 2003; Abraira and Ginty, 2013). While lamina II and III are composed almost, if not exclusively, of interneurons. Functionally, interneurons can be divided into excitatory and inhibitory cells (Todd, 2010). Excitatory interneurons synthesize glutamate as their main neurotransmitter and can be detected by vesicular glutamate transporter 2 (VGLUT2) immunoreactivity in their axons (Todd et al., 2003). Inhibitory interneurons synthesize γ- aminobutyric acid (GABA) or glycine as their main neurotransmitters and can be identified by vesicular GABA transporter (VGAT), and glutamate decarboxylase (GAD67) (Mackie et al., 2003; Todd, 2010). Recently, the dorsal horn excitatory and inhibitory interneurons have been also categorized immunohistochemically by the expression pattern of the homeobox genes T-C-Leukemia homeobox 3 (Tlx3) (Cheng et al., 2004; Xu et al., 2008b), and paired box gene2 (Pax2) (Batista and Lewis, 2008) respectively.

Subpopulations of dorsal horn interneurons have also been identified based on their electrophysiological properties and neurochemical markers. The identification of these distinct populations of interneurons has been instrumental in uncovering the dorsal horn circuits and their contribution to pathological pain (Miraucourt et al., 2007; Duan et al., 2014; Petitjean et al., 2015; Peirs and Seal, 2016). Of particular interest for this dissertation are the populations of opioid-expressing interneurons. MOR interneurons are expressed in excitatory interneurons (Kemp et al., 1996) in lamina IIi and II_o (Corder et al., 2017; Wang et al., 2018) and are thought to be part of a polysynaptic circuit involved in the inhibition of ascending inputs via projection neurons in lamina I and V (Eckert et al., 2003; Chen et al., 2005). Similarly, DOR-containing interneurons are mostly excitatory and located at

the ventral aspect of lamina II_i. However, emerging evidence indicates that DOR and MOR are co-expressed in a few subsets of interneurons, and they internalize and function independently (Wang et al., 2018).

The expression of KOR in spinal circuits still debatable, IHC studies suggest that they are expressed in inhibitory interneurons. Dynorphin, the KOR endogenous ligand, is present in both excitatory and a subset of inhibitory interneurons of the superficial dorsal horn (Todd, 2010; Boyle et al., 2017). In the setting of inflammatory pain, inhibitory-expressing dynorphin interneurons play a central role in the mechanical gate circuitry precluding the development of mechanical allodynia (Duan et al., 2014). However, spinal dynorphin peptide exerts not only anti-nociceptive, but also pro-nociceptive actions (Podvin et al., 2016). For example, in preclinical models of neuropathic pain and chronic inflammation, upregulated spinal dynorphin promotes hyperalgesia possibly via an opioid-independent mechanism (Tan-No et al., 2004; Lai et al., 2006; Luo et al., 2008).

2.3.3 Ascending pathways from dorsal horn to the brain

Nociceptive signals from the dorsal horn are transmitted to the brain via projection neurons located within lamina I and the deep dorsal horn. The projection neurons are mostly concentrated in lamina I and are nociceptive specific. Conversely, the deep dorsal horn contains nociceptive specific and wide-dynamic range neurons (WDR) that are sensitive to both innocuous and noxious stimuli (Braz et al., 2014). Immunohistochemical studies identified that the vast majority of lamina I and some deep dorsal horn projection neurons are neurokinin 1 receptor (NK1) positive cells that are activated upon release of SP from peptidergic nociceptors (Mantyh et al., 1995; Lawson et al., 1997; Todd et al., 2002).

From lamina I and V projection neurons, noxious inputs travel to the contralateral side and ascend to higher brain centers via the spinothalamic and spinoreticulothalamic tracts **(Figure 2.1.B)**. The NK1 positive cells form synapses with neurons within the lateral parabrachial nucleus (PBN), rostral ventral medulla (RVM), and periaqueductal gray (PAG) and send projections to six distinct nuclei in the thalamus (Todd et al., 2002; Craig, 2003). From the thalamus, third order neurons convey to the primary (S1) and secondary (S2) somatosensory cortices, where conscious perception is established, and the discriminative aspects of pain are recognized. Alternatively, the PBN sends connections to limbic structures promoting modulation of the affective and contextual facets of pain (Ossipov et al., 2014).

2.4 Nociceptive plasticity in the dorsal horn

As previously highlighted, the dorsal horn is a primary integrative center for transduction and processing of nociceptive information. Thus, the spinal nociceptive circuits are under the constant influence of afferent inputs and modulation from descending controls to adapt to potential damage. It is generally recognized that tissue injury leads to peripheral sensitization and increased responsiveness of dorsal horn nociceptive neurons to normal and sub-threshold afferent inputs, a process termed central sensitization (IASP), that is implicated in the development of persistent pain states (Latremoliere and Woolf, 2009; Sandkuhler and Gruber-Schoffnegger, 2012).

Central sensitization relies on NMDA receptor (NMDAR) activity. For instance, perioperative non-selective NMDA antagonist administration reduces postoperative pain in rodents (Pogatzki et al., 2000; Shen et al., 2016) and patients (Stubhaug et al., 1997). At the cellular level, NMDAR activation leads to increased cytosolic calcium (Ca^{2+}) and

subsequent activation of several downstream substrates such as Ca^{2+}/calmodulin-sensitive adenylyl cyclases (Zhuo, 2012), and the kinases PKA, PKC and extracellular signal-regulated kinase ($ERK_{1/2}$). These kinases modulate the activity of glutamate ionotropic receptors and trigger transcriptional events (Kawasaki et al., 2004; Latremoliere and Woolf, 2009). The consequent increased excitability and efficacy of dorsal horn synapses following injury results in a central amplification response and enlargement of receptive fields which leads to pain from normally innocuous stimuli (allodynia) and heightened pain sensitivity to noxious stimuli (hyperalgesia) at neighboring non-injury sites (secondary hyperalgesia) that are commonly observed in chronic pain conditions (Woolf, 2007, 2011).

In this work, we used a series of manual von Frey filaments with incremental force increases to evaluate punctate hyperalgesia or allodynia (Chaplan et al., 1994). As the distinction between allodynia and hyperalgesia in rodent models of pain is challenging, the term hypersensitivity is commonly applied throughout this dissertation. In addition, we evaluated nociceptive plasticity in the dorsal horn using immunofluorescence of phosphorylated ERK (pERK), as a marker of central sensitization.

2.5 Latent central sensitization as a model to investigate the transition from acute to chronic pain states

The central sensitization of dorsal horn nociceptive neurons induced by tissue trauma can persist beyond healing and recovery from behavioral signs of hyperalgesia. This persistent spinal sensitization is outweighed by tonic activity of the inhibitory systems in a silent form of neuroplasticity named latent sensitization (LS) (**Figure 2.2**) (Solway et al., 2011; Taylor and Corder, 2014; Walwyn et al., 2016). Previous studies indicate that sensitization of spinal neurons arising from surgical incision facilitates the recruitment of

opioid receptors (Nagakura et al., 2008). Likewise, compelling data from our laboratory indicate that LS is maintained in a remission phase by mu opioid receptor constitutive activity (MOR_{CA}). For example, administration of MOR inverse agonists elicited behavioral signs of withdrawal and hyperalgesia when given 21 days after hindpaw injection of Complete Freund's Adjuvant (CFA) or paw incision, but not in sham animals. Moreover, this injury-induced spinal sensitization lasts at least several months under the inhibitory control of mu opioid receptor systems (Corder et al., 2013).

Interestingly, LS can be transiently unmasked by Gi/o-protein receptor antagonists (Campillo et al., 2011; Solway et al., 2011; Walwyn et al., 2016), chronic administration of opioid agonists (Celerier et al., 2001; Li et al., 2001; Lian et al., 2010) and stress (Rivat et al., 2007; Le Roy et al., 2011; Romero et al., 2017; Chen et al., 2018a). Therefore, LS models resemble episodic chronic pain conditions triggered by, i.e., stressors or subsequent injury and that are characterized by periods of remission. Thus, it may represent a critical stage of pain vulnerability. In summary, these studies suggest that sustained signaling of endogenous opioid receptor systems exert a crucial role in the modulation of pain after injury, and any impairment in these protective systems may result in persistent pain states. Therefore, LS is a robust, highly repeatable phenomenon, well characterized in rodents (Taylor and Corder, 2014; Marvizon et al., 2015) and observed in a subset of human subjects (Pereira et al., 2015b; Pereira et al., 2015a). As such, it is a suitable model to investigate the poorly understood cellular and molecular mechanisms involved in the transition from acute to pathological pain states.

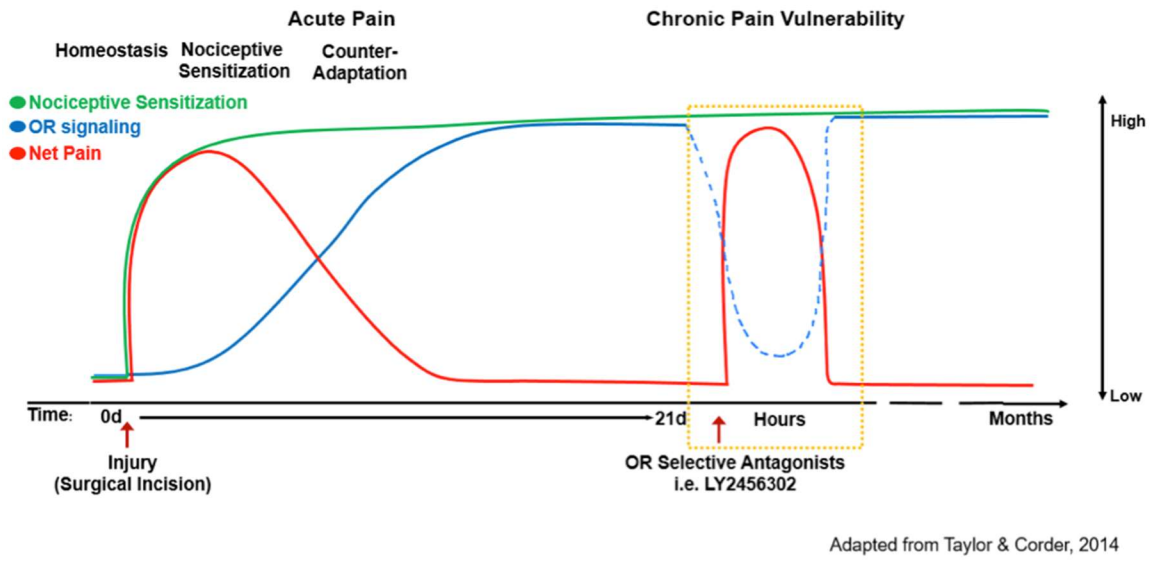

Figure 2.2 Schematic summarizing the sequence of events that occur after injury, leading to a dynamic relationship between the systems that regulate analgesia and pain.

Severe tissue injury triggers the development of latent nociceptive sensitization (**LS, green line**). A compensatory kappa-opioid receptor-mediated (**KOR, blue line**) analgesia silences LS, contributing to pain resolution (**pain, red line**). If the balance is disturbed, i.e., blockade of tonic KOR activity (**dotted line**), LS manifests as spinal neuronal sensitization and pain reinstatement. [Adapted from Taylor & Corder, 2014].

To help answer some of these questions, we employed a translational model of chronic postoperative pain in mice, where a paw incision is performed, and numerous behavioral signs of hyperalgesia are observed for 2-3 weeks (Pogatzki and Raja, 2003). After the recovery of mechanical thresholds, the spinal nociceptive system is challenged with either selective opioid antagonists and/or drugs targeting key pain modulatory proteins, leading to the reinstatement of hyperalgesia (**Figure 2.2**). In chapter 3, we investigate the spinal opioid receptor systems that counterbalance the sustained pronociceptive inputs in the dorsal horn following surgical incision of the hindpaw. In chapter 4, we designed experiments to uncover cellular signaling pathways underlying LS after surgery and to investigate mechanisms by which spinal kappa opioid receptor activity suppresses postoperative pain.

A major gap in the literature is that most animal studies on postoperative pain focused on males. Although, women report high levels of postoperative pain (Storesund et al., 2016), respond to opioid drugs differently than man (Gear et al., 1996a; Gear et al., 1996b), and are at higher risk to develop persistent pain (Kehlet et al., 2006). Therefore, we included female animal cohorts to assess putative sex differences in both arms: endogenous opioid receptor analgesia and the persistent neuroplastic changes (LS) associated with the induction and maintenance of chronic pain after surgery.

2.6. Mediators of spinal nociceptive transmission

2.6.1 Adenylyl cyclase, isoform 1 (AC1)

Adenylyl cyclase type I (AC1) is a membrane-bound calcium/calmodulin-sensitive enzyme that catalyzes the synthesis of cyclic adenylyl monophosphate (cAMP) in mammals. AC1 has been implicated in the modulation of synaptic plasticity and pain (Wu

et al., 1995; Liauw et al., 2005; Wei et al., 2006; Zhuo, 2012; Corder et al., 2013), and it is primarily expressed in the post-synaptic membrane of central and adrenal neurons and retinal cells (Xia et al., 1993; Xia and Storm, 1997; Conti et al., 2007; Wang et al., 2011). AC1 links NMDA activity-dependent increases of intracellular calcium and downstream cAMP signaling pathways, functioning as a key protein for the induction of pain-related plasticity in the CNS. Consequently, AC1 pharmacological or genetic manipulation does not alter nociception but may impact the development of persistent pain states (Vadakkan et al., 2006; Zhuo, 2012). For example, animals receiving systemic administration of the selective AC1 inhibitor, NB001, and AC1KO mice show reduced mechanical hyperalgesia after peripheral inflammation or nerve injury (Wei et al., 2002; Wang et al., 2011; Corder et al., 2013; Griggs et al., 2017). Moreover, AC1 mutant mice exhibit reduced spinal LTP induced by co-administration of serotonin and cAMP-induced by forskolin in the spinal cord slices of adult mice (Wang and Zhuo, 2002), and reduced activation of ERK signaling following peripheral inflammation (Wei et al., 2006). Similarly, AC1 is required for the development of hyperalgesia in the late stages of a preclinical model of chronic musculoskeletal pain (Vadakkan et al., 2006).

Conversely, AC1 is tonically inhibited by opioids and other Gi/o–coupled receptors. Cumulative evidence from our laboratory indicates that AC1 is necessary for the maintenance of LS (Corder et al., 2013). In summary, activation of AC1 leads to increased intracellular cAMP levels that target downstream effector proteins engendering sustained nociception. In Chapter 3, we investigate the hypothesis that KOR signaling opposes LS following surgical incision via prolonged inhibition of AC1 and one or both cAMP target proteins: protein kinase A (PKA) and exchange protein activated by cAMP (Epac). We

also explore potential sex differences in these c-AMP-mediated pronociceptive pathways of LS.

2.6.2 Protein Kinase A (PKA)

Protein phosphorylation is a crucial mechanism for the regulation of nociceptive signal transduction and transmission in the pain pathways. Protein kinase A (PKA) is a classic cAMP-dependent kinase (Walsh et al., 1968) that has been implicated in the regulation of nociception and pain-related synaptic plasticity in the spinal dorsal horn (Sandkuhler, 2007; Latremoliere and Woolf, 2009). PKA is a heterotetrameric complex composed of two regulatory and two catalytic subunits. It is activated upon binding of cAMP to its regulatory domains that lead to dissociation of the catalytic subunits, which in turn activate a myriad of intracellular substrates (Cheng et al., 2008).

Recent evidence from our laboratory indicates that PKA contributes to LS after peripheral inflammation and nerve injury (Fu et al., 2019). PKA contributes to the enhancement of spinal synaptic plasticity via phosphorylation of glutamate ionotropic receptors following peripheral inflammation or robust noxious stimuli. For example, phosphorylation of (alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) AMPA receptors GluR1 subunits leads to increased receptor trafficking and membrane insertion (Fang et al., 2003; Latremoliere and Woolf, 2009). Similarly, PKA-related phosphorylation of the NR1 subunit of NMDAR (Zhou et al., 2000) enhances receptor function and leads to the development of hyperalgesia (Caudle et al., 2005; Gao et al., 2005). Taken together, these findings indicate that activation of PKA after injury potentiates the expression and function of the postsynaptic ionotropic glutamatergic receptors, neurotransmitter release

and spinal synaptic transmission, and possibly contributing to enhancing persistent pain states (Aley and Levine, 1999; Araldi et al., 2015, 2016).

2.6.3 Exchange protein directly activated by cAMP (Epac)

AC1 - cAMP signaling can also modulate nociceptive plasticity via PKA-independent pathways. Epacs (Epac, isoform 1 and Epac, isoform 2) are highly conserved cAMP sensor proteins that act as guanine nucleotide exchange factors for small GTPases (de Rooij et al., 1998; Bos, 2003, 2006). Epac 1 is ubiquitously expressed, while Epac 2 is primarily neuronal (Kawasaki et al., 1998). Epacs are activated as cAMP binds to the regulatory motif (CNB) leading to its dissociation from the catalytic region and subsequent activation of Rap GTPases (Chen et al., 2013). In vitro and in vivo studies indicate that Epacs mediate mechanical hyperalgesia (Wang et al., 2013; Singhmar et al., 2016); following nerve injury (Singhmar et al., 2018) and persistent inflammation through activation of protein kinase C (PKC) in small, non-peptidergic fibers (Griffin, 2005; Hucho et al., 2005). Furthermore, peripheral inflammation enhances the expression of Epacs and subsequent up-regulation of multiple PKC isoforms in the DRG (Gu et al., 2016). Similarly, recent evidence suggests that Epac 1 and Epac 2 are activated during LS induced by surgical incision (Matsuda et al., 2017). Altogether, these results indicate that Epacs are key modulators in the development of persistent pain states, and therefore may represent a promising therapeutic target for pain management.

2.6.4 Phosphorylated Extracellular Signal-Regulated Kinase (pERK)

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPKs) family which is important for intracellular signaling transduction. The ERK isoforms 1 (ERK1) and 2 (ERK2) are expressed in the spinal cord and are

activated in the dorsal horn following tissue injury or inflammation. ERK phosphorylation is found most abundantly in lamina I and II, where most nociceptor afferents relay and NK1 neuronal body are located, and are scattered in lamina III to V of the spinal cord (Ji et al., 1999; Zhuang et al., 2005; Ji et al., 2009).

Tissue injury promotes the activation of multiple neurotransmitter systems and kinases that leads to ERK phosphorylation and contributes to neural plasticity and nociceptive sensitization. For example, peripheral inflammation leads to the release of SP and glutamate, which in turn actives neurokinin 1 receptor (NK1R) or AMPAR and NMDAR. The subsequent rise of intracellular Ca^{+2} elicits the activation of AC1 and several cAMP effectors including PKA and PKC. All these substrates are reported to contribute to ERK1/2 activation *in vivo* and in *ex vivo* spinal cord slices (Ji et al., 1999; Kawasaki et al., 2004; Wei et al., 2006; Chen et al., 2018c). Once activated, pERK perpetuates the phosphorylation and trafficking of glutamatergic receptors (Kohno et al., 2008; Ji et al., 2009), and contributes to CREB phosphorylation and transcription of genes such as c-Fos and prodynorphin (Kawasaki et al., 2004). Thus, pERK contributes to both induction and maintenance of central sensitization, and possibly the pronociceptive component of latent sensitization (Chen et al., 2018c).

In naïve animals or baseline conditions, intense noxious stimuli are necessary to activate ERK, while low threshold or mechanical stimuli (i.e., light touch) are sufficient for ERK activation after injury (Wang et al., 2004; Gao and Ji, 2010). Moreover, pERK expression is increased after Therefore, in chapter 3 and 4 we will evaluate pERK immunoreactivity in the dorsal horn, as a marker of central sensitization, which is detectable 5-10 minutes after nociceptive insult (i.e., pharmacological blockade of opioid

receptors) (Gao and Ji, 2009) followed by innocuous (i.e., light touch) stimuli (Ji et al., 2009; Corder et al., 2013) in post-incisional and sham animals.

2.7 Endogenous opioid receptor systems and pain

The opioid receptor system consists of four highly conserved seven-transmembrane G-protein coupled receptors (GPCRs), the classic mu (MOR), delta (DOR), kappa (KOR), and the closely-related family nociceptin opioid peptide (NOP) receptors. These receptors are ubiquitously expressed in the peripheral tissues and central neurons (Al-Hasani and Bruchas, 2011) (**Figure 2.3**), neuroendocrine and immune cells. All four receptor subtypes were characterized molecularly and pharmacologically (**Table 2.1**). Upon binding of endogenous ligands (beta-endorphin, enkephalin, dynorphin, and nociception/orphanin FQ) and/or exogenous agonists they exert potent antinociceptive effects (Yaksh, 1987; Mansour et al., 1988; Yaksh et al., 1988). The ligands bind to open pockets composed by the extracellular and transmembrane domains. All four receptor subtypes structures were initially characterized in an inactive form upon binding of selective antagonists. Remarkably, MOR and KOR were recently identified under their active state (Manglik et al., 2016; Che et al., 2018). This configuration facilitates the development of new compounds and has been the subject of excitement in the field of opioid pharmacology (Manglik et al., 2012; Manglik et al., 2016).

Opioid receptor activation at different nervous system loci modulates spinal nociceptive transmission via stimulation of G protein-dependent and independent signaling pathways. All four receptor subtypes are coupled to a heterotrimeric inhibitory Gαi/o─Gβγ protein complex. Ligand binding or increased constitutive activity (Corder et al., 2013; Polter et al., 2017) alters the opioid receptor configuration, where $G\alpha_{i/o}$ and $G\alpha_{\beta\gamma}$ subunits

dissociate, leading to the inhibition of adenylyl cyclase - cyclic adenosine monophosphate (cAMP) - dependent signaling pathways (Childers, 1991; Childers et al., 1992; Reisine and Bell, 1993). As a result, the opening of voltage-gated Ca^{2+} channels (VGCC) is reduced (Dickenson et al., 1987; Sullivan et al., 1989; Kohno et al., 1999; Kondo et al., 2005), decreasing neuronal excitability and neurotransmitter release. Additionally, at the post-synaptic membrane $G\alpha_{\beta\gamma}$ complex increases G protein-coupled inwardly rectifying potassium channel (GIRK) conductance, which further hyperpolarizes the neuron (Logothetis et al., 1987). Collectively, opioid receptor agonists decrease neuronal transmission and promote robust antinociception (For comprehensive review please see (Al-Hasani and Bruchas, 2011; Corder et al., 2018).

This dissertation will focus primarily on the pharmacological effects of antagonists of the classic opioid subtypes (MOR, DOR, and KOR, **Table 2.2**) in unmasking postoperative LS and putative sex differences in opioid receptor analgesia (Chapter 3).

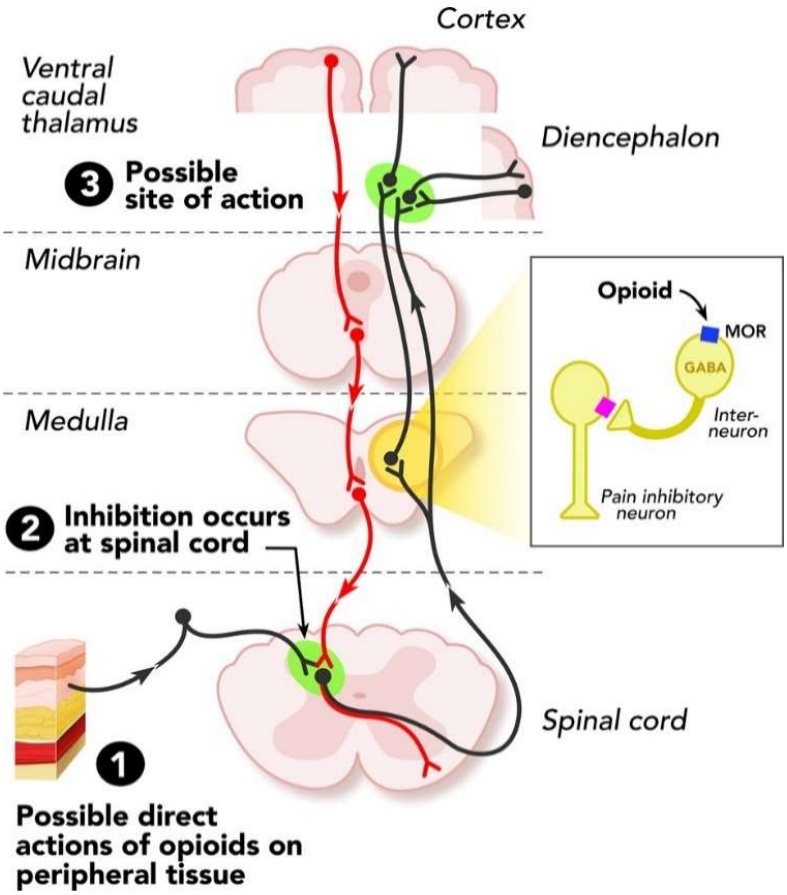

Figure 2.3. Loci of analgesic actions of opioids in pain pathways.

Opioid receptors are expressed throughout the nociceptive pathways. Endogenous and exogenous opioids exert their analgesic actions in the peripheral tissues (**1**), pre- and post-synaptically in the spinal cord (**2**), and brain (**3**) (**black arrows, pain transmission = ascending pathways**); and in descending modulatory tracts from the brainstem - midbrain periaqueductal grey (PAG) and rostral ventromedial medulla (RVM) (**red arrows, pain modulation = descending pathways**). (Adapted from Al Hasani & Bruchas, 2011).

Table 2.1 Opioid receptor systems gene and pharmacological characterization.

The classic opioid subtypes' and respective genes with endogenous and exogenous ligands.

[Adapted from (Puig and Montes, 1998; Dickenson and Kieffer, 2013)].

Opioid Receptor Subtype	MOR	DOR	KOR
Gene	OPRM1	OPRD1	OPRK1
Endogenous Peptide Precursor	Pro-opiomelanocortin (p-POMC)	Pro-enkephalin (p-PENK)	Pro-dynorphin (p-Dyn)
Endogenous Agonists	β-endorphins Enkephalins	Enkephalins	Dynorphins
Agonists	Codeine	Codeine	Codeine
Selective Agonists	DAMGO Morphine	Deltorphin DPDPE	Enandoline Salvinorin A (N) U50,488H U69,593
Antagonists/ Inverse Agonists	Naloxone *Naltrexone	Naloxone *Naltrexone	Naloxone *Naltrexone
Selective Antagonists	*CTOP	*Naltrindole *TIPPψ	CYM51317 GNTI (L) JDTIC (L) *LY2456302/ *(CERC 501) *Nor-BNI (L) Zyklophin

* Drugs used in this dissertation.

L= long-acting; N= natural product.

2.7.1 Delta opioid receptors

Delta opioid receptors (DOR) were the first opioid receptor subtype cloned (Evans et al., 1992; Kieffer et al., 1992). Similarly to MORs and KORs, they are highly conserved across species (Knapp et al., 1995). DORs regulates both nociception and opioid tolerance. Their analgesic effects at the spinal cord level were confirmed in studies using DOR KO mice (Zhu et al., 1999) and pharmacological manipulations. A distinct feature of DOR is that it is found mostly in intracellular structures (Cahill et al., 2001) of sensory neurons and traffic to the cell surface in response to agonist administration (Gendron et al., 2006), chronic inflammation or opioid administration (Cahill et al., 2003). In contrast, recent studies using DOR-GFP mice revealed a relatively high expression of DORs at the neuronal membrane under physiological conditions (Scherrer et al., 2009; Wang et al., 2018).

Several lines of evidence suggest that DORs exert their analgesic actions, at least in part, due to heterodimerization with MORs (Gomes et al., 2000; Kieffer and Gaveriaux-Ruff, 2002; Gendron et al., 2006). Although, recent studies using DOR-GFP indicate that despite significant (~50%) co-localization with MORs in projection neurons in lamina I, they are expressed mostly in distinct populations of interneurons and function independently (Wang et al., 2018). In addition, DORs are present in the non-peptidergic and mechanosensory myelinated nociceptors that project to the skin and are involved in mechanical hypersensitivity in chronic pain states (Scherrer et al., 2009; Bardoni et al., 2014). Thus, DOR activation is involved in the control of mechanical pain. Moreover, microinjection of DOR agonists in the rostral ventral medulla (RVM) leads to

antinociceptive effects in chronic inflammatory models of pain, indicating a supraspinal descending inhibitory control of DORs (Hurley and Hammond, 2000).

2.7.2 Mu opioid receptors

MOR is the main target of most opiate drugs available to treat severe pain. It promotes robust antinociceptive effects once activated by its endogenous peptides β-endorphin or exogenous ligands such as morphine. However, prolonged use of opioid agonists are associated with multiple severe adverse effects including tolerance, dependence, respiratory depression and opioid induced hyperalgesia (Al-Hasani and Bruchas, 2011).

MORs are broadly expressed throughout the pain pathways. Their expression is enhanced in unmyelinated peptidergic fibers or thermonociceptors (Vetter et al., 2006; Chen and Pan, 2008; Bardoni et al., 2014) possibly involved in the control of postoperative pain. The presence of MOR in these afferent fibers also contributes to the development of opioid tolerance and hyperalgesia (Araldi et al., 2015; Corder et al., 2017; Araldi et al., 2018). In the spinal dorsal horn, MOR is present in lamina I projection neurons and subpopulations of lamina II interneurons (Corder et al., 2017; Corder et al., 2018; Wang et al., 2018). In the setting of injury, spinal MORs are thought to promote long-lasting endogenous analgesia via increased constitutive activity (Corder et al., 2013) predominantly in central terminals of primary afferents (Severino et al., 2018).

Spinal nociceptive transmission is highly modulated by MOR actions in supraspinal sites as well. Microinjections of MOR agonists or electrostimulation of the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM), key modulatory areas in the brainstem, promotes robust antinociception. These effects are attributed to MOR inhibition

of excitatory ("on cells") or disinhibition of inhibitory ("off cells") in the RVM that send projections directly to the dorsal horn (Basbaum and Fields, 1984; Ossipov et al., 2014). Recent studies identified two distinct populations of "off cells" as GABAergic neurons co-expressing Penk (proenkephalin) to inhibit nociceptive transmission in the dorsal horn, and a population of MOR-expressing cells that synapses with Penk-positive interneurons in the dorsal horn that modulates mechanonociception presynaptically (Francois et al., 2017; Corder et al., 2018). Therefore, MOR is a potent endogenous analgesic system promoting antinociceptive effects in different sites of the nervous system.

2.7.3 Kappa opioid receptors

KOR is highly expressed in all levels of the pain neuroaxis and descending modulatory circuits. Under physiological conditions, KOR is activated upon binding of its endogenous peptide ligand, dynorphin (Goldstein et al., 1979; Goldstein et al., 1981; Fischli et al., 1982), which is expressed and released from nerve terminals in the CNS to selectively act on peripheral or central KORs (Chavkin et al., 1982; Wagner et al., 1991; Chavkin, 2013). Depending on the site of action, the activation of the KOR-dynorphin system is implicated in numerous physiological processes and pathologies including analgesia (Millan et al., 1985; Spampinato and Candeletti, 1985; Clemente et al., 2004; Auh and Ro, 2012; Snyder et al., 2018), paradoxical hyperalgesia (Tan-No et al., 2002; Lai et al., 2006; Luo et al., 2008), stress (Knoll and Carlezon, 2010; Chavkin, 2013; Xie et al., 2017), mood disorders (Land et al., 2008; Carroll and Carlezon, 2013; Li et al., 2016), and reinstatement of drug seeking (Sun et al., 2010; Jackson et al., 2015; Reed et al., 2018). When activated by dynorphin or exogenous full or partial agonists, KOR promotes analgesic effects via Gi/o signaling (Chavkin, 2011), while its aversive and psychomimetic

effects are mediated by activation of mitogen-activated protein kinase MAPK (Bruchas et al., 2007a; Land et al., 2008) in the brain. Emerging evidence indicates a possible dynorphin-independent KOR activation after stress (Polter et al., 2017) and injury (Walwyn et al., 2016).

2.7.3.1 Peripheral

In the periphery, KOR is present in free nerve terminals in the enteric mucosa and skin to regulate neuroendocrine, immune functions (Hedner and Cassuto, 1987), and pain (Joris et al., 1987; Stein et al., 1988; Moon et al., 2016; Snyder et al., 2018). In the setting of inflammation, endogenous opioids are released from immune cells in a P38 MAPK dependent process (Stein, 1991). Endogenous and exogenous opioids may penetrate the perineurial barrier of nociceptors after inflammation to interact with opioid receptors and promote analgesia (Stein, 1991; Antonijevic et al., 1995; Rittner et al., 2009). Alternatively, there is speculation that KOR agonists may interact with receptors expressed on immune cells (Snyder et al., 2018). The peripheral antinociceptive effects of KOR exogenous agonists are well characterized. KOR activation attenuates neurogenic inflammation via reduction of plasma extravasation and hyperemia induced by inflammatory mediators (Snyder et al., 2018). Additionally, it decreases thermal (Joris et al., 1987), and mechanical sensitivity after chronic inflammation (Auh and Ro, 2012). A recent study characterized the expression of KOR in peptidergic nociceptors in the skin and visceral tissues and implicated peripheral KOR in the modulation of mechanical pain associated with surgical incision (Snyder et al., 2018).

2.7.3.2 Spinal Cord

KOR is highly abundant in the superficial dorsal horn (lam. I and II) of the spinal cord (Harris and Drake, 2001; Harris et al., 2004). It is present in the central terminals of a subset of primary peptidergic afferent neurons in mice and humans, low threshold mechanoreceptors and the dorsal column (Snyder et al., 2018). The expression of KOR in projection and interneurons is not yet well characterized. KOR agonists delivered spinally elicit analgesia in several pain models (Milan, 1990) and suppress chloroquine induced-itch (Kardon et al., 2014). Similarly, the release of dynorphin from interneurons located in the superficial dorsal horn reduce mechanical pain (Todd, 2017). Moreover, dynorphin peptide i.t. administration leads to analgesia in acute pain models, an effect that is mediated by KOR binding (Spampinato and Candeletti, 1985; Podvin et al., 2016).

Conversely, in chronic inflammatory and nerve injury models dynorphin administration elicits hyperalgesia/allodynia via non-opioid pathways, possibly through direct or indirect interaction with NMDA or bradykinin receptors (Vanderah TW et al, 1996; Tan-No K, et al., 2002; Lai et al., 2006, Luo MC et al., 2008). The effects of KOR agonists in spinal nociceptive plasticity (LTP) yielded mixed results; some studies failed to demonstrate an effect of KOR agonists in spinal field potentials (Buesa et al., 2008), while others found KOR inhibitory effects on C-evoked potentials (Sullivan and Dickenson, 1991) and voltage-gated Ca+2 currents (Snyder et al., 2018). Moreover, systemic Norbinaltorphimine (Nor-BNI) administration three weeks after surgical incision under remifentanil restored mechanical hyperalgesia in mice. In line with these results, spinal administration of long-lasting KOR antagonists also elicited hyperalgesia three weeks after intraplantar CFA injection in rodents, when the mechanical thresholds returned

to baseline levels. It is important to note that the long-lasting KOR antagonists used in the abovementioned studies have a delayed onset (Munro et al., 2012), off-target effects in MOR (Horan et al., 1992) in high dosages, and are thought to act indirectly on KOR through JNK-1 signaling, which in turn leads to KOR desensitization (Bruchas et al., 2007b). Therefore, the precise effects of spinal KOR activation in chronic pain are still unresolved.

Thus, throughout this work, we will investigate the effects of spinal KOR in chronic postoperative pain using primarily the selective reversible short-acting antagonist LY2456302 (currently known as CERC 501), which acts directly on KOR (Rorick-Kehn et al., 2014b). Moreover, the vast majority of animal research focuses on the KOR effects mediated by $G_{\beta\gamma}$ or β-arrestin-independent signaling. Therefore, we further expand the literature evaluating antinociceptive effects of kappa in chronic pain via components of G_{α} (AC1 and cAMP) signaling.

2.7.3.3 Brain

KORs are expressed in multiple brain loci. The dynorphin-KOR system is implicated in the regulation of several brain psychopathologies. For example, KOR regulation of the mesocorticolimbic circuitry underlies the etiology of mood disorders, dysphoria and reinstatement of drug seeking (Cahill et al., 2014) through inhibition of monoaminergic centers at several sites (Lalanne et al., 2014). The amygdala is an important site for regulation of stress, and affective components of pain (Narita et al., 2006; Cahill et al., 2014; Xie et al., 2017), while the RVM is a key site of modulation in inflammatory pain. Microinjection of KOR agonist in the left central amygdala in rats “primed” with sumatriptan infusion reveals reduction of paw mechanical thresholds, which is reversed by

KOR blockers, implying pronociceptive actions of KOR (Xie et al., 2017; Navratilova et al., 2018). Conversely, microinjection of KOR agonists in the RVM reduces mechanical and thermal sensitization, effects that became more evident as inflammatory pain persists (Hurley and Hammond, 2000; Schepers et al., 2008b; Schepers et al., 2008a). Therefore, the modulatory effects of KOR systems in pain processing is dependent on the site of action and regulatory functions.

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CHAPTER 3. Sex Differences in Kappa Opioid Receptor Inhibition of Latent Postoperative Pain

This chapter is a similar version of the manuscript submitted to Neuropharmacology. Donahue R.R., Lambert J., Smith B., and Taylor B.K. are additional authors of the manuscript.

3.1 Abstract

Tissue injury induces an allostatic state of latent pain sensitization (LS) in the dorsal horn neuron that is opposed in part by mμ-opioid receptor constitutive activity. Blockade of this endogenous inhibitory activity with naltrexone or naloxone reveals LS as a reinstatement of mechanical hypersensitivity. However, naltrexone or naloxone also bind with significant affinity to delta and kappa opioid receptors. Therefore, to evaluate the specific contribution of mu, delta, and kappa opioid receptors to endogenous inhibition of LS, we performed plantar incision at the hindpaw, waited 21 days for the resolution of hyperalgesia, and then intrathecally injected subtype-selective antagonists. We found that neither of the two delta receptor-selective antagonists naltrindole (1μg) nor TIPP[Ψ] (1-10μg) changed mechanical thresholds. By contrast, the mu receptor-selective antagonist CTOP (1-1000ng) dose-dependently reinstated mechanical allodynia, to a similar degree in male and female mice. Similarly, the kappa receptor-selective antagonists Nor-BNI (0.1-10μg) and LY2456302 (0.1-10μg) reinstated mechanical allodynia. Furthermore, LY2456302 (10μg) increased the expression of phosphorylated signal-regulated kinase (pERK), a marker of central sensitization, in dorsal horn neurons but not glia. Sex studies revealed that a lower (0.3μg) but not higher dose (10μg) of LY2456302 reinstated

hypersensitivity to a greater degree in female as compared to male mice. Our results suggest that spinal MOR and KOR, but not DOR, maintain LS within a state of remission to reduce the intensity and duration of postoperative pain and that endogenous KOR but not MOR analgesia is greater in female mice.

3.2 Introduction

Tissue injury induces a sustained form of neuronal plasticity, termed latent sensitization (LS), that is kept within a state of remission by compensatory pain inhibitory systems that include opioid, neuropeptide Y and alpha2-adrenergic receptor signaling (Rivat et al., 2002; Campillo et al., 2011; Solway et al., 2011; Corder et al., 2013; Taylor and Corder, 2014; Walwyn et al., 2016). Long-lasting mechanisms of endogenous opioid receptor analgesia include mμ-opioid receptor constitutive activity (MOR_{CA}) (Corder et al., 2013; Walwyn et al., 2016); evidence for endogenous opioid inhibition of LS exists not only in mice and rats, but also in human experimental pain models (Pereira et al., 2015b; Pereira et al., 2015a). Some studies also indicate a contribution of delta-opioid receptor (DOR) and kappa-opioid receptors (KOR) (Campillo et al., 2011; Walwyn et al., 2016; Xie et al., 2017). However, these studies were limited to behavioral indices of pain and/or animals received prior administration of a different drug. To address these deficiencies, we performed plantar incision at the hindpaw, waited 21 days for the resolution of hyperalgesia, and then intrathecally injected multiple doses of multiple subtype-selective opioid receptor ligands. In addition to behavior, we also assessed touch-evoked changes in the expression of phosphorylated extracellular signal-regulated kinase (pERK), a marker of central sensitization of nociceptive neurons in the dorsal horn (Gao and Ji, 2009).

Numerous studies report sex differences in the ability of *exogenous* KOR ligands to modulate pain in both rodents (Mogil et al., 2003; Terner et al., 2003a; Terner et al., 2003b; Sternberg et al., 2004; Lomas et al., 2007; Auh and Ro, 2012; Robinson et al., 2016) and humans (Gear et al., 1996a; Gear et al., 1996b; Pande et al., 1996b; Pande et al., 1996a; Gear et al., 1999). However, questions of sex differences in *endogenous* opioid receptor analgesia have not been rigorously studied. To address this gap, we investigated whether sex is a main factor in endogenous spinal MOR- and KOR-mediated inhibition of LS by testing both male and female mice.

3.3 Materials and Methods

3.3.1 Subjects

Experiments were carried out in 8-12 weeks old male and female C57Bl/6 mice (Charles Rivers Laboratories, Inc, Wilmington, MA). Mice were housed maximum 5 same-sex littermates per cage in a temperature and humidity-controlled room (14:10hr light-dark cycle, lights on at 6:00 am) with ad libitum access to food and water. The Institutional Animal Care and Use Committee at the University of Kentucky, protocol (#2017-2768), approved all procedures following American Veterinary Medical Association guidelines. Mice were acclimated to the colony housing room for several days and then handled for 2 days by the experimenter before the initiation of the studies.

3.3.2 Plantar Incision Model of Postoperative Pain

Post-operative pain was induced by longitudinal incision of the plantaris muscle, as previously described (Pogatzki and Raja, 2003; Jang et al., 2011). Under isoflurane anesthesia (5% induction and 1.5-2% maintenance, via a nose cone) and antisepsis of the left hind paw with Chlorascrub® then alcohol, a #11 scalpel blade was used to perform a

5mm through the skin and fascia using, beginning 2mm from the proximal edge of the heel extending towards the digits. The underlying muscle was raised with curved forceps, extended 4 mm and incised longitudinally, leaving the origin and insertion of the muscle intact. The overlying skin was closed with synthetic 5-0 sutures (PDS*II, Ethicon), followed by application of antibiotic ointment was applied. Surgery was typically completed within 5-10min. Sutures were removed on the post-operative day 10. Sham controls received anesthesia but no surgical incision.

3.3.3 Direct Intrathecal (i.t.) Drug Delivery

As previously described (Fairbanks, 2003), non-anaesthetized mice were lightly restrained in a towel, and a 30G x1/2 needle (Becton Dickinson) attached to a 25-µl Hamilton micro syringe, was inserted into the subarachnoid space between the L5/L6 vertebrae. The needle was angled about 30-45 degrees with the horizontal plane and slightly advanced until reflexive tail flick was confirmed, then 5 µl of drug or vehicle was slowly administered. The needle was held in place for 30sec, to ensure the solution fully enters the spinal canal, withdrawn, and then the mouse was returned to its testing chamber.

3.3.4 Drug Dosing

All Drugs or vehicle were intrathecally injected in a 5 µl bolus. The MOR selective ligand D-Phe-c[Cys-Tyr-Trp-Orn-Thr-Pen]-Thr-NH2 (CTOP, Cat.#1578 Tocris) was dissolved in sterile saline (0.9%NaCl) and injected at doses of 1 – 1000ng (Gendron et al., 2007; Corder et al., 2013). Based on these doses we established dose response curves. The DOR-selective ligand Naltrindole HCL (Sigma-Aldrich N115) was dissolved in sterile saline and injected at doses of 0.1-10µg (Scherrer et al., 2009), H-Tyr-TicΨ [CH2-NH] Phe-Phe-OH ($TIPP_{[\Psi]}$, gift from NIDA Drug Supply Program) was sonicated in ddH_20 at

30°C for 30 minutes and injected at doses of 1-10µg (Riba et al., 2002b; Riba et al., 2002a). Doses of naltrindole equal to or greater than 10 µg and TIPP[Ψ] at 20 µg elicited adverse effects such as itching, licking and tail biting, so we limited their use to a high dose of 1 µg of naltrindole and 10 µg of TIPP[Ψ]. The KOR-selective ligand norbinaltorphimine dihydrochloride (Nor-BNI, 0347 Tocris) was dissolved in saline and injected at doses of 0.1-10µg. For Figures 3.4 and 3.6, LY2456302 (Rorick-Kehn et al., 2014a; Rorick-Kehn et al., 2014b) was obtained from the NIDA Drug Supply Program, sonicated in ddH_20 at 30°C for 30 minutes, and 0.1-10µg was injected. For Figures 3.6, 3.7 and 3.8, LY2456302 was obtained from Med Chem Express, dissolved 1:1:8 in EtOH: Alkamuls EL-620, Rhodia, Cranbury, NJ: saline, and injected at dose of 10 µg.

Testing began at day 21 after surgery to allow for mechanical thresholds to return to pre-incision baseline levels. At this time, animals were assigned to experimental groups by a laboratory colleague (RRD, WF) who kept the code until the experiments were completed. On day 21, some animals received appropriate drug, or vehicle. After a seven-day washout, animals given drug on day 21 were given vehicle or higher dose of that same drug on day 28. Conversely, animals given vehicle on day 21 were given drug on day 28. This cross-over design was not used with the KOR ligands due to their extended half-lives.

3.3.5 Pain-Like Behavioral Assessment: Stimulus-Evoked

All animals were acclimated in a temperature and light controlled room within individual Plexiglas boxes placed on the top of a stainless-steel mesh platform for 30 to 60 min prior to behavioral testing. Mechanical thresholds were assessed using an incremental set of 8 von Frey monofilaments (Stoelting, Illinois), ranging in gram force from 0.008g to 6g. The filaments were applied perpendicularly to the ventral surface of the hindpaw lateral

to the suture site with enough force to engender slight bending of the filament. A positive response was characterized as a rapid withdrawal of the paw away from the stimulus fiber within 5s. Left and right hindpaws were tested alternately. Using the up-down method (Chaplan et al., 1994), response patterns were logarithmically converted into 50% withdrawal thresholds (in gram force). As previously described, animals were randomly assigned to experimental groups in all studies. Investigators (LCP and RRD), blinded to experimental groups by a research associate, and conducted all behavioral experiments.

3.3.6 Touched-evoked Phosphorylated Extracellular Signal-Regulated Kinase (pERK)

As previously described (Corder et al., 2013), 21 days after surgery mice received either vehicle or LY2456302 (10µg). Two hours after injection, mice were lightly anesthetized with isoflurane (1.5%), and the ventral surface of the ipsilateral hindpaw was mechanically stimulated with gentle 2-second stroke with a cotton swab from heel to toe. This was repeated every 5s for 5min. After an additional 5 min pause, mice were deeply anesthetized with isoflurane and transcardially perfused with cold 0.01M phosphate buffer saline (PBS, Fischer Scientific) /heparin buffer (10,000 USP units/L), followed by 10% phosphate formalin buffer. Lumbar spinal cords were harvested and post-fixed in the same fixative overnight at 4ºC, and then cryoprotected with 30%sucrose until total submersion (1-3 days).

3.3.7 Histology and Immunohistochemistry

Transverse sections (30µm) from L3-L5 were obtained on a sliding microtome (Leica, SM 2000R). A series of sections, each 240µ apart, was washed in 0.01M PBS, blocked in 3% normal serum (goat or donkey; Gemini Bioproducts), containing 0.3%

Triton X-100 (Sigma Aldrich) in 0.01M PBS for 1h, and then incubated with primary rabbit antibody anti-phosphorylated-ERK ½ antiserum (1:800, Cell Signaling) at 4ºC for 36 hours on a shaker (Fig.3.4, 3.6 and 3.7), or goat anti- -Iba1 (isoforms 1 and 3) (1:500, Sigma Aldrich), and/or chicken anti-GFAP (1:1000, Abcam) at 4ºC overnight. The following day, sections were again washed in 0.01M PBS and incubated for 1h at room temperature with secondary conjugated antibodies (1:1000, Invitrogen: donkey anti-rabbit Alexa Fluor 568, donkey anti-goat Alexa Fluor 488, goat anti-rabbit Alexa Fluor 568, and/or goat anti-chicken Alexa Fluor 488. For neuronal co-staining, FITC-conjugated mouse NeuN (MAB377X 1:500, EMD Millipore) was incubated for 1h at room temperature (Fig.3.8). The sections were washed in 0.01M phosphate buffer, mounted and cover slipped with mounting medium with DAPI (Vectashield, Vector laboratory). At least five good quality sections from segment L4 were selected from each subject for microscopy.

3.3.8 Microscopy

Fluorescence Microscopy. For quantification of pERK immunoreactivity, low magnification images taken with Nikon TE-2000 microscope equipped with a 10X objective were captured and analyses with NIS-Elements Software AR 4.13.05. An examiner blinded to treatment and sex counted the number of positive pERK cells in laminae I-II and III-V.

Confocal Microscopy. For double-label immunohistochemistry, confocal images were acquired with a Nikon A1 Confocal Microscope System equipped with a 60X oil-immersion objective (numerical aperture 1.49) located in the Microscopy Core at the Department of Biochemistry at the University of Kentucky Biomedical Biological Sciences

Research Building. Laser excitation and emission windows for the different fluorophores were as follows: Alexa Fluor 568 (Cy3) – excitation 543.5 nm, emission 543-633 nm, Alexa Fluor 488 (EGFP) – excitation 488nm, emission 483-543nm, and DAPI - excitation 405nm, emission 423-483nm. Z-stacks images were obtained with 0.5mm slice thickness. Images were processed using Nikon NIS-elements confocal software AR 4.30.02.

3.3.9 Statistical Analysis

Mechanical thresholds are summarized as mean values quoted with the corresponding standard error (mean ± SEM). The effects of Drug and Time were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni multiple correction tests. Data were then re-plotted as area under the curve using the trapezoidal method and integrative thresholds were analyzed using one-way or two-way ANOVA as appropriate, followed by Bonferroni tests using Prism 8 (GraphPad, La Jolla, CA). Non-linear regression analyses of Maximum Possible Effect (%MPE) were used to determine the ED50 for each drug. %MPE was calculated as follows: % MPE = 100* (post-injection threshold – preinjection threshold) / (post-injury threshold – pre-injection threshold). For Figures 3.5 and 3.6, sex differences in mechanical threshold were analyzed using repeated measures three-way ANOVA, with Sex and Drug analyzed as between-subject factors, and Time analyzed as a within-subject factor. To assess the Drug by Sex interactions, linear mixed models were used with random intercepts and time slopes for each animal. Pairwise time points were individually analyzed using the mixed model approach to increase the power to detect Sex by Drug interactions. Linear Mixed models were fit using the *lme4 V1.1-15* package in *R V3.4.3* (R software). The criterion for the level of significance was set at $P \leq 0.05$ (★). All graphs were created using Prism 8 (GraphPad, La Jolla, CA).

3.4 Rationale and Results

3.4.1 MOR and KOR, but not DOR opioid receptor signaling provide postoperative endogenous analgesia

To determine which opioid receptors provide endogenous analgesia in the setting of LS, we performed plantar incision, waited 21 days for mechanical hypersensitivity to completely resolve, and then to these mice, termed PIM 21d mice, we intrathecally injected opioid receptor subtype-selective antagonists.

Mu opioid receptor. As illustrated in **Figure 3.1A**, intrathecal administration of the selective MOR antagonist CTOP in male PIM 21d mice dose-dependently reinstated mechanical hypersensitivity as compared to saline ($F(28,217) = 9.43$, $p < 0.0001$). The pronociceptive effect of CTOP was robust, peaking at 30 min (0.14 ± 0.03g, 0.08 ± 0.02g at 1ng and 1000ng respectively), and lasted for at least 180 min. As illustrated in **Figure 3.1B**, area under the curve analysis (AUC 30-180min) illustrates concentration-dependent effects of CTOP ($F(4, 29) = 41.3$, $p < 0.0001$). As illustrated in **Figure 3.1C**, conversion of the 30 min timepointn data to %MPE yielded an ED50 value of 1.84 ng.

Delta opioid receptor. As illustrated in **Figure 3.2A**, this dose of naltrindole did not change mechanical hypersensitivity in male PIM 21d mice ($F(6,156) = 2.01$, $p = 0.07$). We then tested an alternative DOR antagonist ($TIPP_{[\Psi]}$ 1µg-10µg), and Fig 2B similarly no effect on mechanical hypersensitivity ($F(12,102) = 0.35$, $p = 0.97$).

Kappa opioid receptor. We used either the prototypical KOR antagonist nor-BNI or the shorter-acting and highly selective KOR antagonist LY24506302 due to its favorable pharmacokinetic properties compared to nor-BNI (Munro et al., 2012; Rorick-Kehn et al., 2014b). As illustrated in **Figures 3.3A-B**, the selective KOR antagonists nor-BNI (0.1-

10µg) or LY2456302 (0.1-10µg) produced a dose-dependent reinstatement of mechanical hypersensitivity compared to saline- (Nor-BNI, $F(27,216) = 3.73$, $p<0.0001$ and LY2456302, $F(33,286) = 5.63$, $p<0.0001$, Fig. 3.3A, D). The effect on hyperalgesia of both Drugs peaked at 3h (Nor-BNI, 0.19 ± 0.06g and LY2456302, 0.30 ± 0.04g) and lasted at least 72h. As illustrated in **Figures 3.3 C-D**, AUC analysis (1-72h) yielded F values of ($F(3, 24) = 12.90$, $p<0.0001$) Nor-BNI and ($F(3, 26) = 20.37$, $p<0.0001$) for LY2456302. As illustrated in **Figures 3.3 E-F,** focus on the 3h timepoint yielded ED_{50} values of 0.87µg for nor-BNI and 1.04µg for LY2456302.

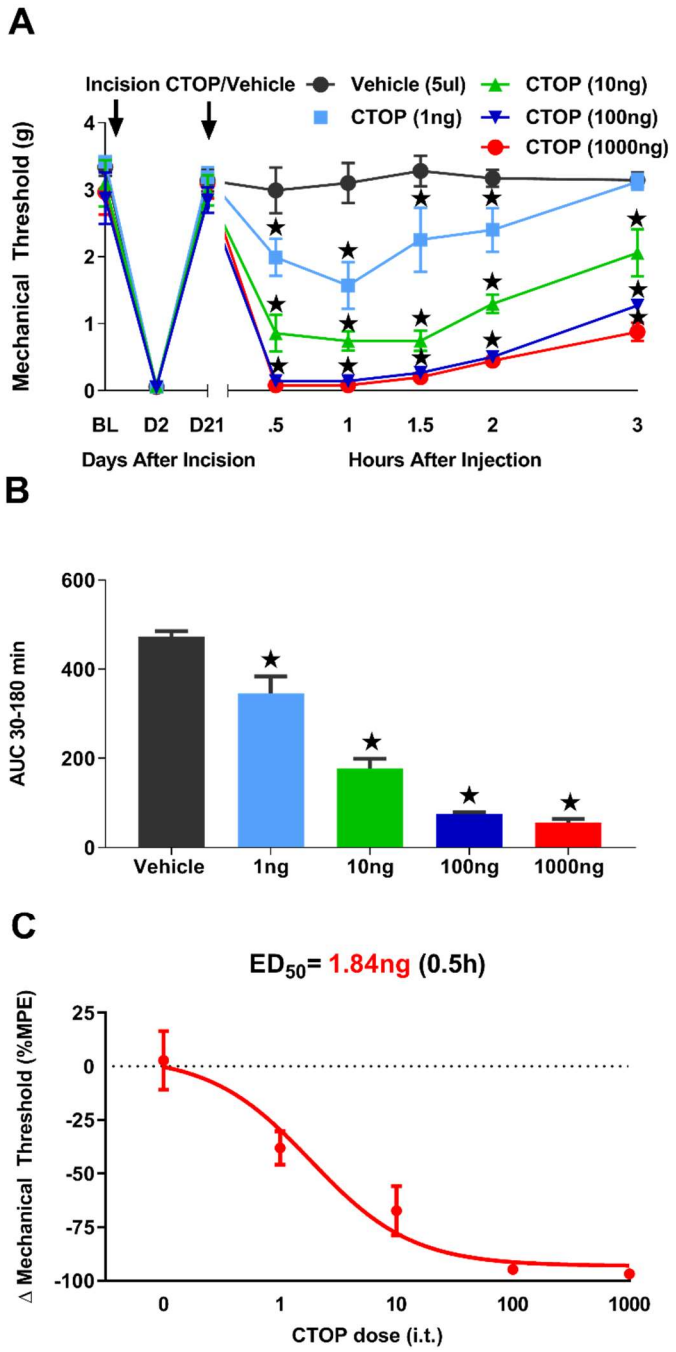
A
Incision CTOP/Vehicle
Vehicle (5ul)
CTOP (10ng)
CTOP (1ng)
CTOP (100ng)
CTOP (1000ng)
Mechanical Threshold (g)
BL
D2
D21
.5
1
1.5
2
3
Days After Incision
Hours After Injection
B
AUC 30-180 min
Vehicle
1ng
10ng
100ng
1000ng
C
ED50= 1.84ng (0.5h)
Δ Mechanical Threshold (%MPE)
CTOP dose (i.t.)

Figure 3.1. Spinal MOR blockade reinstates mechanical hypersensitivity in a dose dependent manner.

The MOR selective antagonist CTOP was intrathecally (i.t.) administered 21d after plantar incision in C57/Bl6 male mice. (**A**) Time course of mechanical hypersensitivity after CTOP (1ng-1000ng) administration produces reinstatement of mechanical hypersensitivity in a dose-dependent manner (n=8 per dose). (**B**) The area under the curve (AUC, 30-180 min) illustrates the concentration-dependent actions of CTOP on mechanical hypersensitivity. (**C**) Dose-response analysis of the data at 0.5h after injection revealed an ED_{50} of 1.84ng. MPE: maximum possible effect. ★$p<0.05$ CTOP vs. vehicle. BL = baseline behavior prior to drug administration.

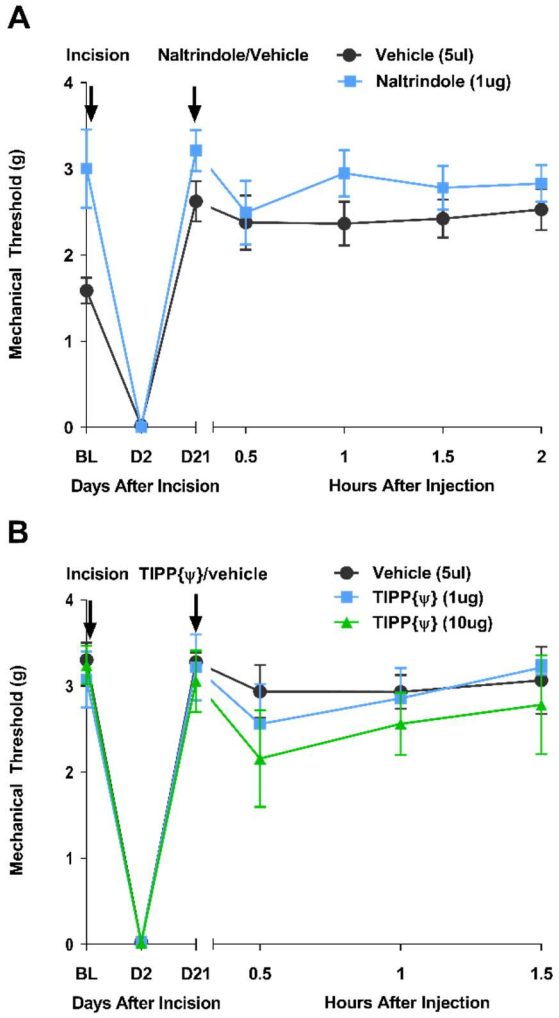

Figure 3.2. Spinal DOR selective antagonists do not reinstate mechanical hypersensitivity.

DOR selective antagonists Naltrindole (**A**) and $TIPP_{(\Psi)}$ (**B**) were administered i.t. 21d after plantar incision in C57Bl6 mice. Time course of mechanical hypersensitivity after Naltrindole (1ug-10ug), and $TIPP_{(\Psi)}$ (1ug-10ug) administration show no significant reinstatement of mechanical hypersensitivity (n=10-18 per dose Naltrindole p=0.07, n=6-8 per dose $TIPP_{(\Psi)}$, p=0.9). BL = baseline behavior prior to drug administration.

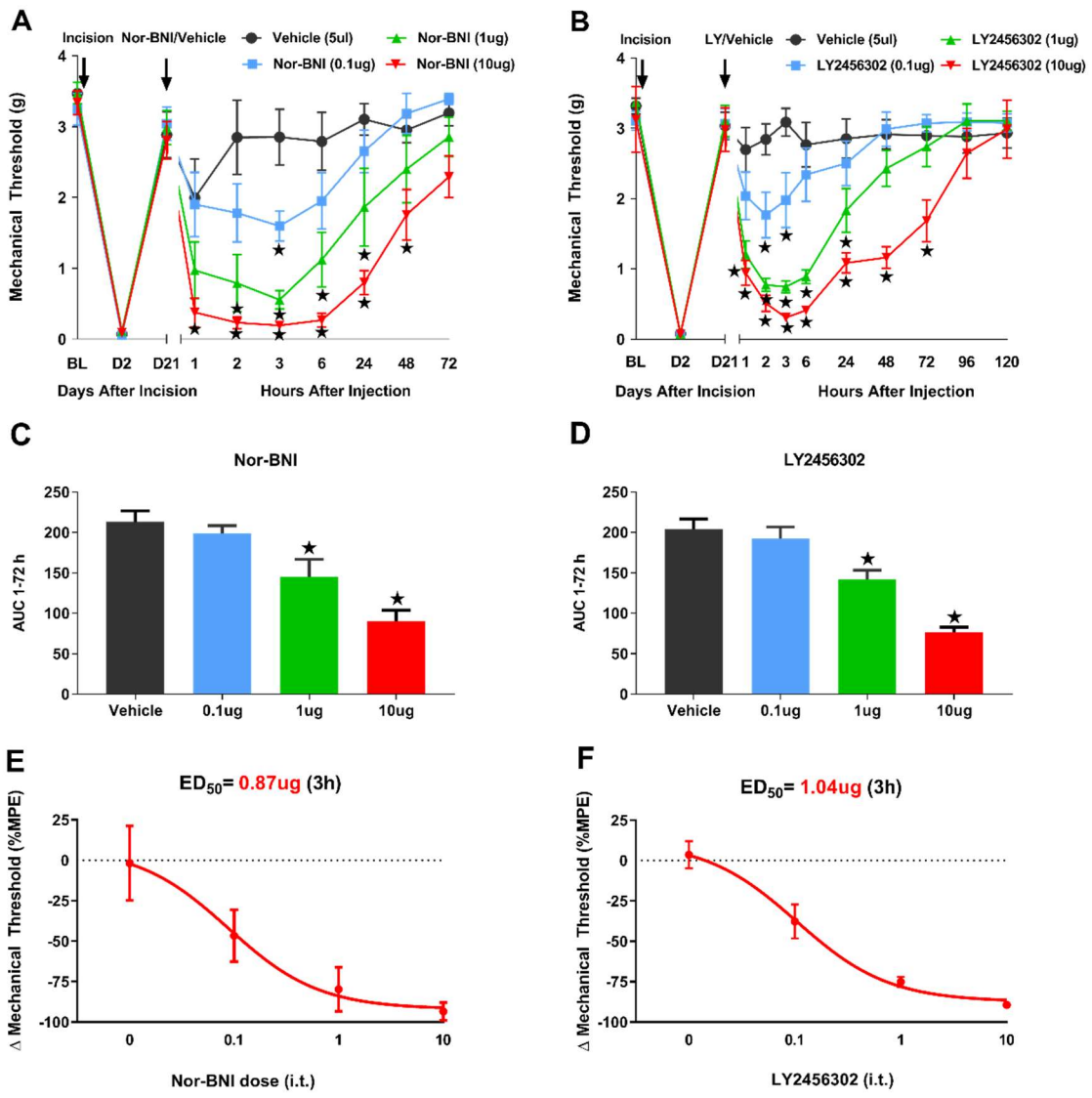

Figure 3.3. Spinal KOR blockade reinstates mechanical hypersensitivity in a dose-dependent manner.

The KOR selective antagonists Nor-BNI **(A)** and LY2456302 (**B**) were intrathecally (i.t.) administered 21d after plantar incision in C5/Bl6 male mice. Time course of mechanical hypersensitivity after Nor-BNI (0.1ug-10ug; n=5-10 per dose) and LY2456302 (0.1ug-10ug; n=6-8 per dose) administration exhibited robust reinstatement of mechanical hyperalgesia in a dose-dependent manner. (**C-D**) Area under the curve (Nor-BNI AUC,

1-72h; LY AUC, 1-120h) illustrates the concentration-dependent actions of both drugs on mechanical hypersensitivity. (**E-F**) Dose-response analysis of the data at 3h revealed an ED_{50} of 0.87ug for Nor-BNI, and an ED_{50} of 1.04ug for LY2456302 for mechanical hypersensitivity. MPE: maximum possible effect. ★$p<0.05$ Ly/Nor-BNI vs. vehicle. BL = baseline behavior prior to drug administration.

3.4.2 Spinal kappa opioid antagonism modulates latent nociceptive sensitization in the dorsal horn

Our pharmacological behavior experiments demonstrated that spinally delivered KOR antagonists produce reinstatement of hyperalgesia 21 days after surgical paw incision. These findings suggest that similar to MOR, KORs exert tonic inhibition that opposes pronociceptive signals within the spinal pathways, preserving LS within a state of remission. Thus, to determine whether KOR activity reduces the cellular manifestations of latent sensitization after incision, we evaluated the light-touch-evoked expression of pERK, a well-recognized marker of central sensitization during pathological pain (Gao and Ji, 2009; Ji et al., 2009), in the superficial (I-II) and deep laminae (III-V) of the dorsal horn. We assessed immunohistochemical changes in pERK expression in the dorsal horn during the peak of drug activity, 2h after LY or saline i.t. delivery. Intraplantar capsaicin was used as a positive control as it activates C-afferent fibers markedly increasing the phosphorylation of ERK in the superficial dorsal horn (Kawasaki et al., 2004). We quantified the number of pERK-positive cells as a marker of activation of the nociceptive sensitization pathway. We found that compared to controls, light-touch of the injured hindpaw increased pERK cell profiles 21 days after plantar incision in the ipsilateral dorsal horn (4.89 ± 0.48 vs. 0.77 ± 0.11, $p<0.0001$; 1.36 ± 0.22 vs. 0.22 ± 0.22, $p=0.3$) LY vs. vehicle in the ipsilateral lam I-II and III-V respectively. (4.89 ± 0.48 vs. 1.52 ± 0.16) LY ipsilateral vs. contralateral paw. $F(3, 16) = 16.83$, $p<0.0001$) 2 hours after KOR blockade (**Figure 3.4 B-E**).

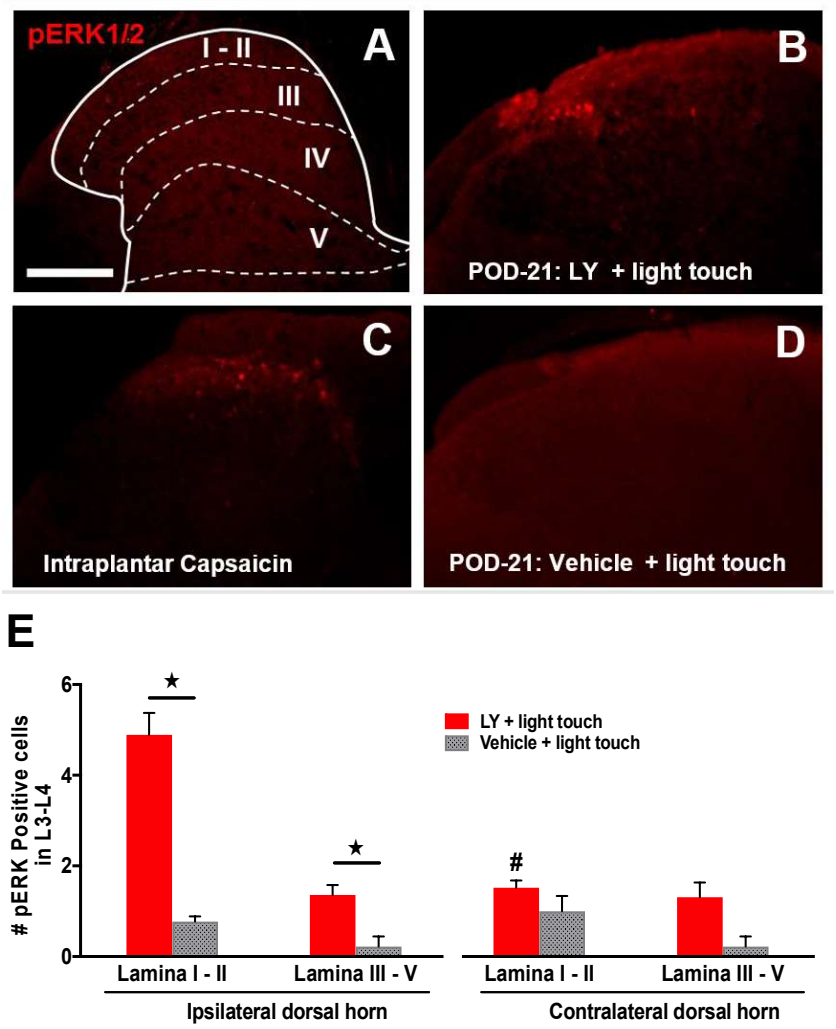

Figure 3.4. KOR spinal blockade via LY2456302 increased light-touch evoked positive pERK cells in the dorsal horn.

The number of positive pERK cell profiles in the dorsal horn **(A)** superficial (I-II) and deep (III-V) lamina the Rexed is increased after intrathecal administration of **(B)** LY (10ug) and **(C)** capsaicin (positive control) compared **(D)** to vehicle administration in 21d postoperative male mice. **(E-F)** Histogram showing light touch-induced pERK-positive cells are significantly higher in LY treated mice compared to controls in the side ipsilateral to the injury (n=3-4, 4-5 slices per animal).★ $p<0.05$ LY vs. vehicle, # $p<0.05$ ipsilateral vs. contralateral. Scale bar 100µm. Figure (**A**) adapted from Corder et al, 2013.

3.4.3 Postoperative endogenous KOR Analgesia is enhanced in female mice

As illustrated in **Figures 3.5 and 3.6**, plantar incision produced a robust decrease in mechanical threshold on the paw ipsilateral but not contralateral to injury. The time course of mechanical hypersensitivity did not significantly differ between male and female mice ($p>0.05$) when tested at either the paw ipsilateral to injury (Fig 3.5.A-B and 3.6.A-B) or contralateral to injury (Fig 3.5C-D and 3.6C-D). Thresholds returned to baseline by post-operative day 21 in each sex. These results are consistent with previous findings in mouse incision models (Banik et al., 2006).

Postoperative patients exhibit sex differences in endogenous opioid inhibition of pain (Fillingim and Gear, 2004). We previously reported that inverse agonist-induced reinstatement of mechanical hyperalgesia and facial grimace in the CFA model of LS did not differ between male and female mice (Corder et al., 2013); however, these studies were limited to to a single dose of naltrexone which can interact with mu, delta, and kappa opioid receptors. Therefore, we asked whether low or high doses of CTOP or LY2456302, as determined by the dose-response curves in Figures 3.1 and 3.3, would yield sex differences in mechanical thresholds after injection in PIM 21d male and female mice.

<u>Mu opioid receptor</u>. Our results in male mice demonstrated that both CTOP 100ng and 1000ng dose produced robust hyperalgesia in PIM 21d male mice (Figure 3.1A), which were close to the maximum efficacy (Figure 3.1C). Thus, we selected 100ng for further studies. As illustrated in **Figure 3.5A**, a high dose of CTOP (100ng) but not vehicle produced robust hyperalgesia in PIM 21 male and female mice at the ipsilateral side (Main Drug Effect $F(1,90)= 57.25$, $p<0.0001$; Time vs. Sex vs. Drug Interaction $F(5,90)= 0.19$, $p=0.96$). As illustrated in **Figure 3.5B**, CTOP (100 ng) also produced a robust hyperalgesia

at the contralateral side that did not differ between the sexes (Main Drug Effect $F(1, 54) = 48.80$, $p < 0.0001$; Time vs. Sex vs. Drug Interaction $F(5, 54) = 0.87$, $p = 0.50$). As illustrated in **Figure 3.5C**, AUC analysis confirmed that CTOP (100 ng)-induced reinstatement of hyperalgesia did not differ between the sexes ($F(3, 43) = 0.90$, $p = 0.78$).

Since this relatively high dose of CTOP may have produced a floor effect that could have masked a sex differences, we repeated this study using the lowest effective dose in males (Figure 1). As illustrated in **Figure 3.5D-F**, low-dose CTOP reinstated hyperalgesia (main Drug effect $F(1,44) = 4.40$, $p = 0.04$ ipsilateral; $F(1,14) = 5.99$, $p = 0.02$ contralateral) to a similar degree in male and female mice (Time vs. Sex vs. Drug Interaction $F(5,220) = 0.24$, $p = 0.94$, Fig. 3.5B; Time vs Sex vs. Drug Interaction $F(5,70) = 0.34$, $p = 0.88$), AUC $F(3, 58) = 0.08$, $p = 0.1$ (**Figure 3.5.F**).

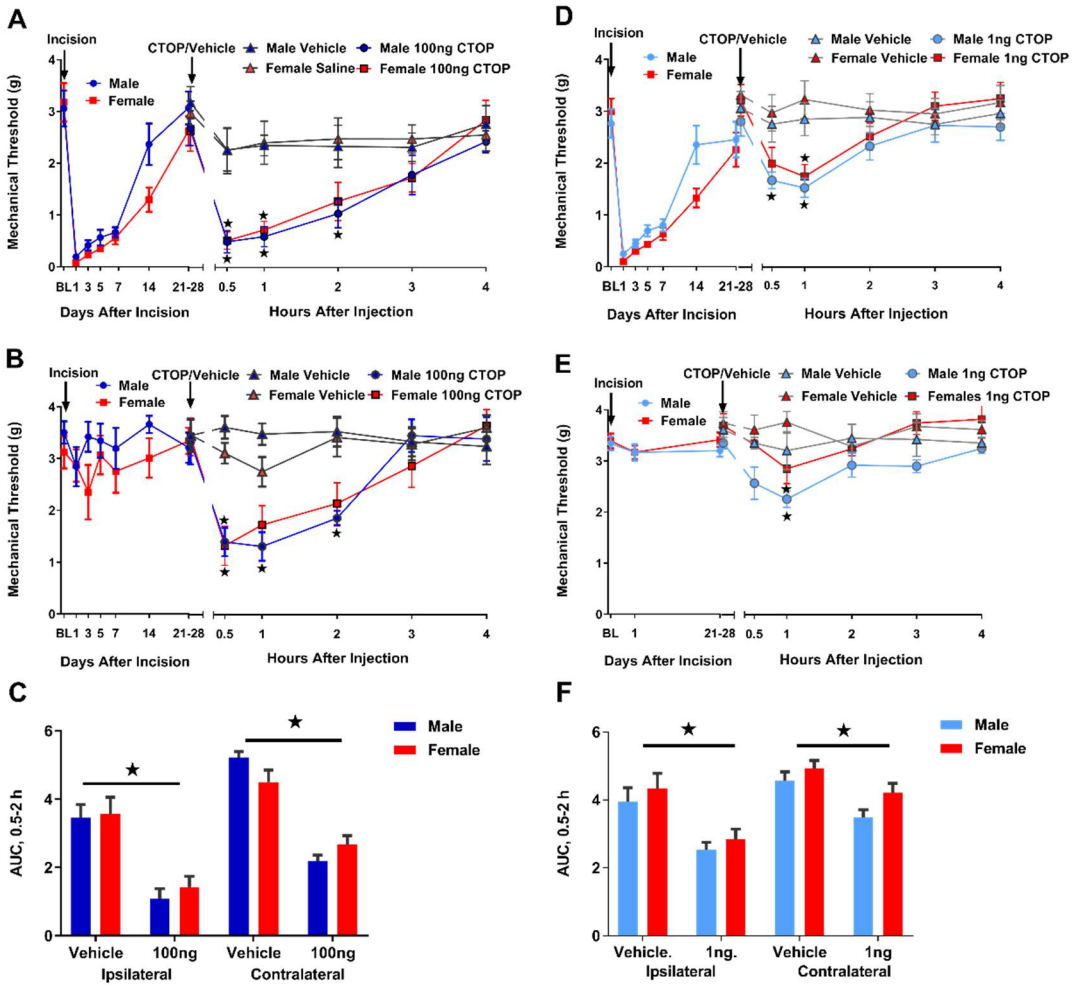

Figure 3.5. Spinal MOR blockade reinstates mechanical hypersensitivity equally in male and female mice.

(A-B) Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7,14 and 21-28 days in male or female C57Bl/6 male, and then after intrathecal injection of CTOP at a dose of 100ng at the paw ipsilateral (A) or contralateral (B) to plantar incision. **(C)** Analysis of area under the curve (AUC, 0.5-2h) illustrates that CTOP (100ng) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. **(D-E)** Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7, 14 and 21-28 days in male or female C57Bl/6 male, and then after intrathecal injection of CTOP

at a dose of 1ng at the paw ipsilateral (D) or contralateral (E) to plantar incision. **(F)** Analysis of area under the curve (AUC, 0.5-2h) illustrates that CTOP (1.0ng) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. n=4-10 per group. ★$p<0.05$ CTOP vs. vehicle. Data are represented as mean +/- SEM.

Kappa opioid receptor. Next, we investigated whether sex differences exist in endogenous KOR-mediated inhibition of postoperative pain in our LS model. We chose not only the prototypical KOR antagonist (inhibitor) Nor-BNI, but due to its delayed onset and duration of action which can last up to four weeks (Horan et al., 1992; Munro et al., 2012), we also selected the shorter-acting KOR antagonist LY24506302 due to its favorable pharmacokinetic properties compared to nor-BNI (Rorick-Kehn et al., 2014a; Rorick-Kehn et al., 2014b). As illustrated in **Figure 3.6A-B**, a relatively high dose of LY24506302 (10µg) reinstated mechanical hypersensitivity compared to saline in male and female mice at the sides both ipsilateral ($F(1, 21) = 21.80$, $p<0.0001$) and contralateral ($F(1, 21) = 14.44$, $p=0.001$) to plantar incision. However, the degree of reinstatement did not differ between the sexes (Time vs. Sex vs. Drug interaction $F(8,168) = 0.13$, $p = 0.99$, 5A; $F(8,168) = 0.25$, $p = 0.25$, 5D), and this is further illustrated in **Figure 3.6C** by area under the curve analysis across the 1 to 120 hr time points ($F(3, 43) = 0.356$, $p=0.78$).

Since this relatively high dose of LY24506302 may have produced a floor effect that could have masked a sex difference, we repeated this study using a lower dose of 0.3 µg as suggested by the dose-response curve in **Figures 3.3D-F**. As illustrated in **Figure 3.6D**, LY2456302 (0.3 µg) reinstated hyperalgesia at the side ipsilateral to incision in both male and female mice, and importantly to a greater extent in female mice (Bonferroni posthoc test $p<0.05$). **Figure 3.6E** illustrates a smaller effect on the contralateral side that did not differ between sexes. Further analysis in **Figure 3.6F** illustrates that female PIM 21d mice exhibited greater mechanical hyperalgesia at the peak of LY-induced reinstatement at the 2-4h time points (Sex vs. Drug Interaction, Satterthwaite: p-value=0.041).

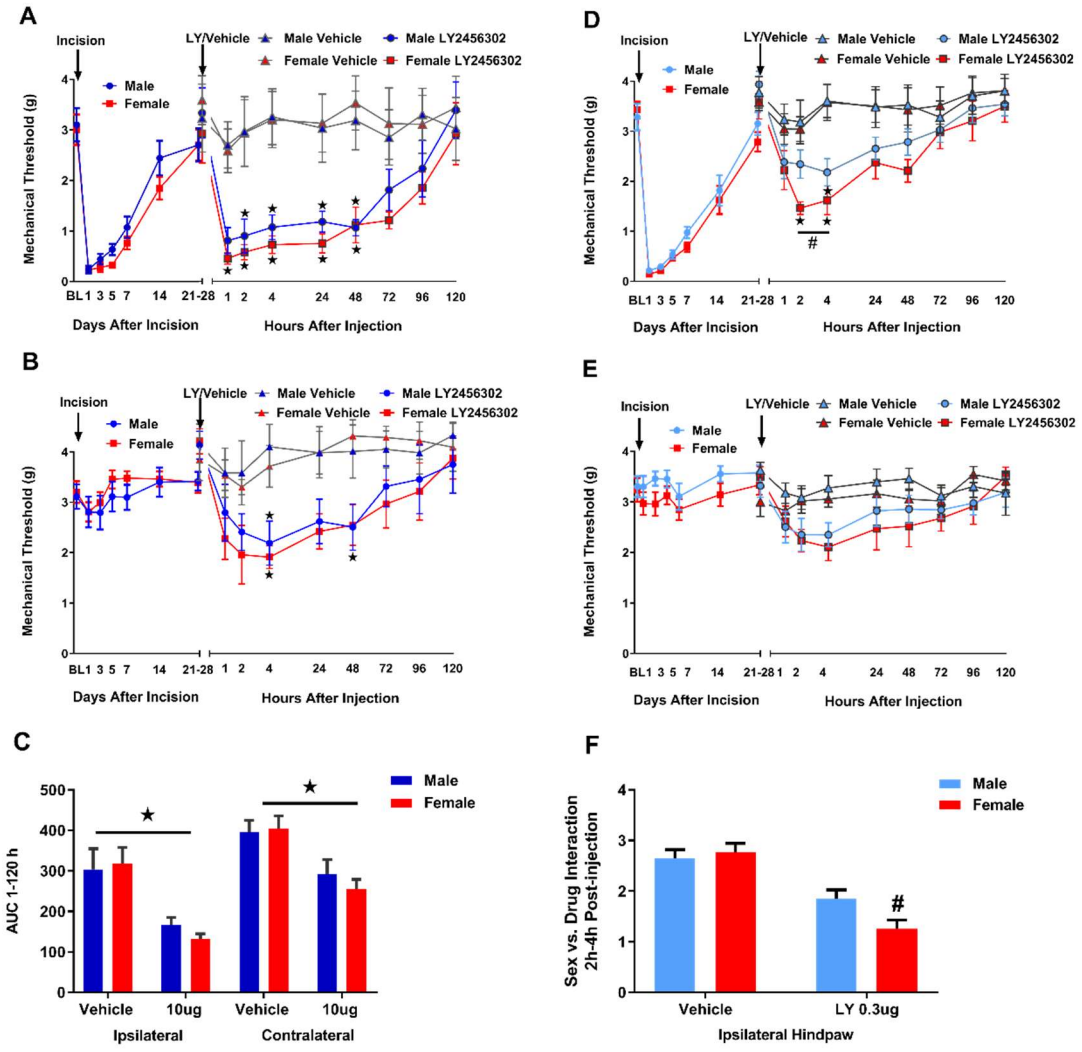

Figure 3.6. LY2456302-induced reinstatement of mechanical hypersensitivity is greater in females.

(A-B) Time course of mechanical thresholds at baseline (BL), 1, 3,5, 7, 14 and 21-28 days in male or female C57Bl/6 male, and then after intrathecal injection of LY2456302 at a dose of 10µg at the paw ipsilateral (A) or contralateral (B) to plantar incision. **(C)** Analysis of area under the curve (AUC, 0.5-2h) illustrates that LY2456302 (10µg) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. **(D-E)** Time course of mechanical thresholds at baseline (BL), 1, 3, 5,

7, 14 and 21-28 days in male or female C57Bl/6male, and then after intrathecal injection of LY2456302 at a dose of 0.3μg at the paw ipsilateral (D) or contralateral (E) to plantar incision. **(F)** Analysis of area under the curve (AUC, 0.5-2h) illustrates that LY2456302 (0.3μg) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. n=6-8 per group. ★$p<0.05$ LY2456302 vs. vehicle, #$p<0.05$ male vs. female. Data are represented as mean +/- SEM.

3.4.4 Tonic KOR inhibition of dorsal horn neuron activity is enhanced in female mice

We previously reported that MOR_{CA} inhibits long-lasting sensitization of dorsal horn neurons induced by cutaneous inflammation (Corder et al., 2013). To begin to address whether KOR inhibits neuronal activity as well, we evaluated light touch-evoked expression of pERK in the superficial (I-II) and deep laminae (III-V) of the dorsal horn, two hours after intrathecal administration of LY2456302 or saline in male and female mice, 21 days after either sham or plantar incision surgery. As illustrated in **Figures 3.7A-B, D-E, G-H**, light touch was associated with only a small number of pERK-positive profiles in sham controls that received LY2456302 or vehicle treated mice. By contrast, **Figures 3.7C**, **3.7F**, and **3.7G** illustrate that LY2456302 evoked a larger number of pERK+ profiles in PIM 21d mice at both superficial lamina I-II ($F(5,23) = 1.61$, $p<0.001$) and deeper laminae III-V ($F(5,44) = 6.60$, $p=0.001$). Importantly, LY2456302 was associated with significantly more pERK+ profiles in laminae I-II in females as compared to males ($p<0.05$). p-ERK can be expressed on neurons, astrocytes, and microglia (Ji et al., 2009). To determine the cell type(s) that express pERK after LY2456302, we co-labelled with NeuN, GFAP, and Iba1. As illustrated in **Figure 3.8**, confocal microscopy revealed that pERK was expressed exclusively in NeuN-positive cells in laminae I–V of the dorsal horn obtained from female (**Figures 3.8A-U**) or male (**Figures 3.8A'-U'**) mice.

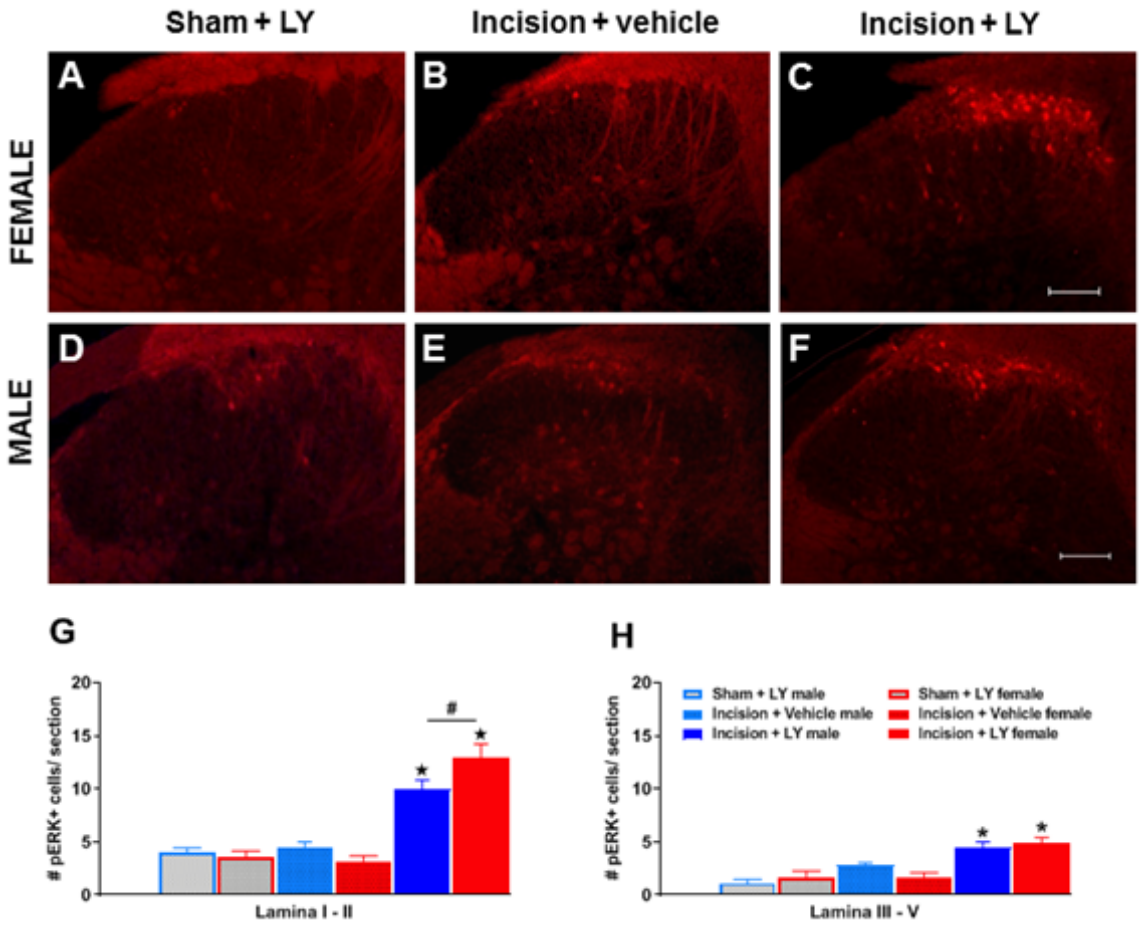

Figure 3.7. LY2456302-evoked pERK^{+} cells in the dorsal horn is greater in female mice.

Representative photomicrographs of light touch-evoked pERK-immunoreactivity in L4 lumbar sections from **(A-C)** female or **(D-F)** male mice, 21 days after sham surgery plus 10µg LY2456302 (A, D), plantar incision plus vehicle (B, E), or plantar incision plus 10µg LY2456302 (C, F). **(G-H)** Quantification of mean number of touch-evoked pERK+ profiles in lamina I-II (G) or lamina III-V (H). Data represent mean +/- SEM. ★$p<0.05$. LY2456302 vs. vehicle and sham incision. *$p<0.05$ LY2456302 vs. shams. # $p<0.05$ n= 4-6 (5 slices per mouse). Scale bar: 100µm.

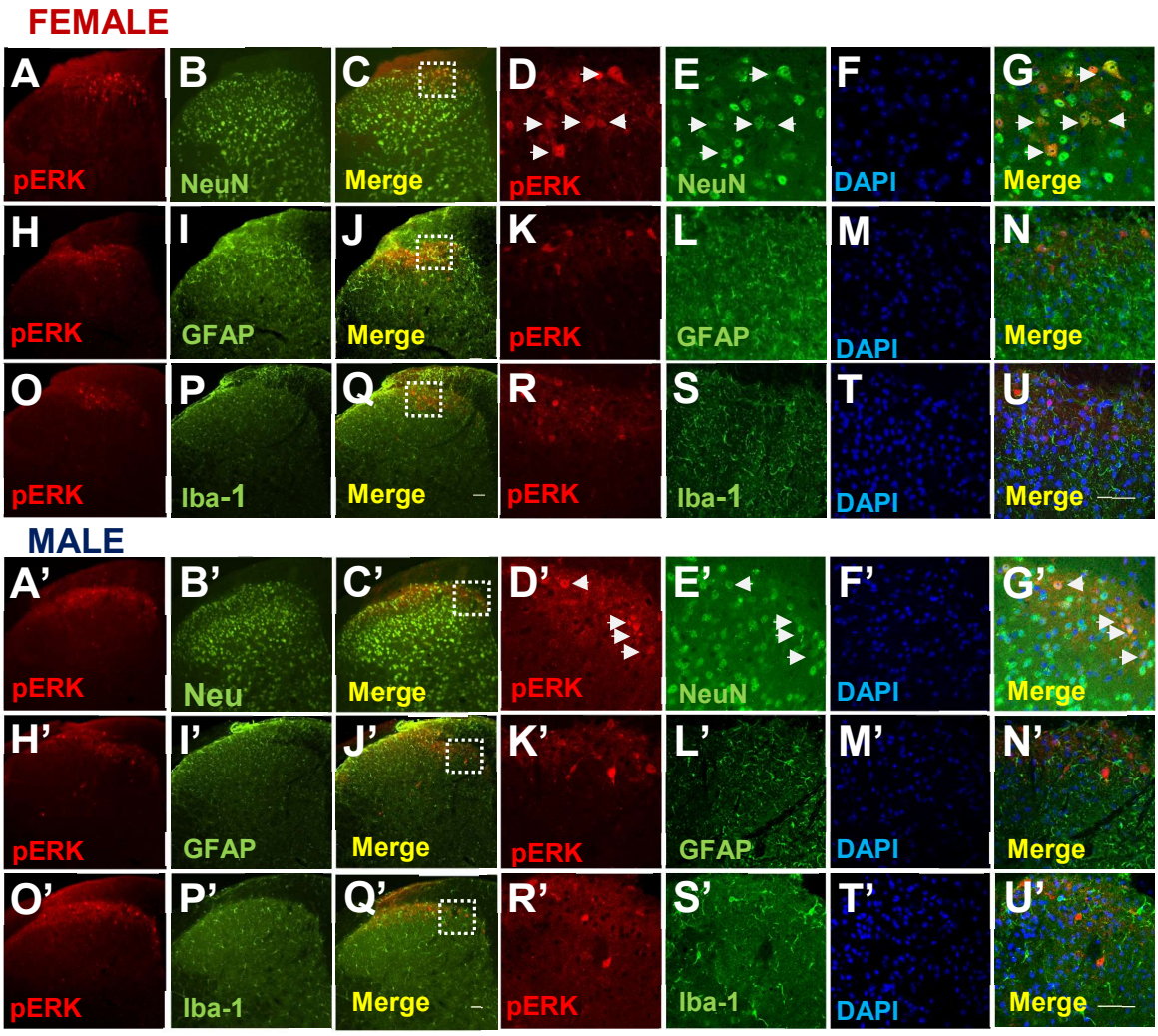

Figure 3.8. Touch-evoked pERK in the dorsal horn is co-localized exclusively with neurons.

To determine whether KOR blockade evoked pERK expression in neurons, microglia, or astrocytes, we co-stained with NeuN, Iba1, or GFAP, respectively. Representative transverse lumbar (L4) spinal cord sections (30µm) from postoperative-21d mice 2h after intrathecal delivery of LY2456302 (10µg) and an ipsilateral light touch paw stimulation. Sections from females were co-labelled with pERK (A, C, D, G, H, J, K, N, O, Q, R and U), and either NeuN (B-C, E, G), GFAP (I-J, L, N), or Iba-1 (P-Q, S-U). DAPI (F-G, M-

N, T-U). Sections from males were co-labelled with pERK (A', C', D', G', H', J', K', N', O',Q', R' and U'), and NeuN (B'-C', E',G'), GFAP (I'-J', L',N'), or Iba-1 (P'-Q', S'-U'). DAPI (F'-G', M'-N', T'-U'). Arrows indicate pERK co-labeling. Light microscopic images in the left three columns were taken with a 10X objective, while confocal images in the right four columns were taken with a 60x objective. Scale bars: 50 µm.

3.5 Discussion

3.5.1 Tonic MOR activity provides long-lasting inhibition of postoperative pain

Tissue injury induces latent pain sensitization (i.e., LS) that is mediated in part by NMDA receptors, AMPA receptors, and adenylyl cyclase 1 signaling mechanisms in the dorsal horn (Corder et al., 2013; Taylor et al., 2019). These pronociceptive mechanisms are kept within remission by opposing pain inhibitory mechanisms (Solway et al., 2011; Corder et al., 2013; Walwyn et al., 2016; Fu et al., 2019) that include MOR_{CA} (Corder et al., 2013; Walwyn et al., 2016). Evidence for the contribution of MOR comes in part from behavioral pharmacology studies using MOR-selective inhibitors. For example, when administered 21 days after the intraplantar injection of complete Freund's adjuvant (after the resolution of initial hyperalgesia), intrathecal administration of a single dose of CTOP reinstated hyperalgesia (Corder et al., 2013). The current results not only extend these findings to the plantar incision model of postoperative pain, but also demonstrate a dose-response curve with an ED50 value of approximately 2 ng.

3.5.2 Spinal DORs do not provide long-lasting inhibition of postoperative pain

Selective DOR activation inhibits mechanical hypersensitivity associated with injury (Scherrer et al., 2009; Gaveriaux-Ruff et al., 2011; Bardoni et al., 2014) either through heterodimerization or synergistic interactions with MOR (Gomes et al., 2000; Hurley and Hammond, 2000; Gendron et al., 2007; Al-Hasani and Bruchas, 2011) or via autonomous mechanisms in distinct populations of spinal neurons, different than those that express MOR (Wang et al., 2018). In contrast to these studies using *exogenous* application of DOR agonists to inhibit early inflammatory pain, the current studies tested the

hypothesis that *endogenous* activation of DOR inhibits the longer-lasting vulnerability to inflammatory pain associated with LS. However, neither of the DOR antagonists that we used, naltrindole nor $TIPP_{[\Psi]}$, significantly changed mechanical threshold when administered during the remission phase of LS.

Our DOR results are not consistent with Walwyn and colleagues who reported that subcutaneous administration of naltrindole reinstated hyperalgesia in a mouse CFA model of LS, and that either intrathecal or subcutaneous naltrindole reinstated hyperalgesia in a rat CFA model of LS (Walwyn et al., 2016). One possibility for the differences observed in the latter study and ours is that we tested the animals at a later time point after DOR administration. However, the reinstatement observed in the latter study was very short-lasting and induced at an earlier time point (15 min after i.t. administration) in both experimental and control groups. Thus, confounders such as anxiety and sensitization of the subjects from repeated pharmacological experiments in the same animal could not be ruled out. Albeit, it is fundamentally interesting to speculate here that the plantar incision model and the CFA model (Walwyn et al., 2016) could induce LS via different receptors or mechanisms.

In summary, our data suggest that DOR inhibitors do not reinstate hyperalgesia, and thus we conclude that spinal DORs do not provide long-lasting inhibition in our model of postoperative latent pain sensitization. Since our studies were limited to the intrathecal route of administration future studies are needed to investigate the role of endogenous DORs expressed at peripheral terminals of primary afferent neurons (Scherrer et al., 2009) or supraspinal sites involved in the descending inhibition of spinal transmission (Hurley and Hammond, 2000).

3.5.3 Tonic KOR activity provides long-lasting inhibition of postoperative pain

The current results demonstrate that the kappa receptor-selective inhibitors nor-BNI and LY2456302 dose-dependently reinstated behavioral signs of postoperative pain, and LY2456302 increased the stimulus-evoked expression of pERK, a marker of central sensitization, in dorsal horn neurons but not glia. Our results are consistent with and extend previous studies that suggest a contribution of endogenous KOR to the inhibition of hyperalgesia. For example, systemic administration of nor-BNI reinstated hyperalgesia after paw incision performed under remifentanil in the mouse (Campillo et al., 2011) Furthermore, Marvizon and colleagues reported that nor-BNI reinstated mechanical hyperalgesia in the CFA model of LS (Walwyn et al., 2016). Prodynorphin deficient mice also responded with reinstatement of mechanical hyperalgesia, consistent with the hypothesis that, like MOR_{CA}, inflammation leads to constitutive activity at KOR (Walwyn et al., 2016). Therefore, in the current studies of LS, we have been careful to describe nor-BNI and LY2456302 as KOR inhibitors, since we do not yet know whether their behavior is indicative of inverse agonism and ligand-independent constitutive activity, or antagonism and ligand-dependent activity. Albeit, our findings indicate that LS is maintained in remission by tonic KOR activity at the dorsal horn.

In addition to the spinal sites of action suggested by our intrathecal injection studies, peripheral and supraspinal sites may also contribute to endogenous KOR inhibition of LS. Regarding peripheral sites, KOR is expressed in the nerve terminals of peptidergic populations of primary afferent neurons arising from the skin, viscera, and synovial tissues, and KOR signaling inhibits nociceptor sensitization and pain (Labuz et al., 2007; Cunha et al., 2012; Moon et al., 2016; Snyder et al., 2018). A recent study indicated that peripherally-

restricted KOR agonists markedly reduced mechanical hyperalgesia at the early inflammatory phase of plantar incision (Snyder et al., 2018), implicating peripheral KOR as a major modulator of acute postoperative pain. Regarding supraspinal sites, microinjection of KOR agonists into the rostral ventral medulla activated descending modulatory neurons to inhibit nociception (Hurley and Hammond, 2000; Schepers et al., 2008b; Schepers et al., 2008a) . Furthermore, the current study (Fig 3.6.) demonstrated that KOR inhibitors reinstate hyperalgesia not only at the side ipsilateral to injury but also at the contralateral side, thus further implicating supraspinal modulation of LS as previously proposed for MOR (Corder et al., 2013) and suggested by the current CTOP results (Fig 3.5).

KOR inhibitors produce not only hyperalgesia as in the current studies, but also antihyperalgesia in other studies. For example, in rats primed with sumatriptan or morphine infusion, delivery of KOR inhibitors in the central amygdala prevented acute stress-induced hyperalgesia. In these studies, the pronociceptive effects of KOR were elicited from a descendent modulatory circuit from the right amygdala via MAPK signaling (Xie et al., 2017). Furthermore, KOR in the central nucleus of the amygdala promotes behaviors suggestive of disinhibition, aversiveness, anxiety (Narita et al., 2006) and pain facilitation (Navratilova et al., 2018). Thus, we propose that the direction of effect depends on the site of modulation, with KOR activation at the central terminals of primary afferent and intrinsic dorsal horn neurons leading to pain inhibition, while KOR activation at certain brain sites seems to cause pain.

3.5.4 Sex differences in postoperative pain: evidence and controversies

Numerous studies have investigated sex differences in nociception (Greenspan et al., 2007; Hendrich et al., 2012) and endogenous opioid pain modulation (Tambeli et al., 2001; Fillingim and Gear, 2004; Fillingim et al., 2009). As described previously (Banik et al., 2006), we found that male and female mice presented similar mechanical thresholds and responded to plantar incision with a mechanical hyperalgesia of similar intensity and time course. However, these results are in contrast with other preclinical studies that indicate that females present lower mechanical nociceptive thresholds (Barrett et al., 2002a; Rasakham and Liu-Chen, 2011; Hendrich et al., 2012; Chartoff and Mavrikaki, 2015) and take longer to recover from injury than males (Vacca et al., 2014). Furthermore, women commonly report more severe pain than men (Greenspan et al., 2007; Tsang et al., 2008; Fillingim et al., 2009) and are more predisposed to develop persistent postoperative pain (Kehlet et al., 2006). Moreover, human laboratory indicate that females present lower mechanical nociceptive thresholds and take longer to recover from injury than males (Riley et al., 1998; Fillingim and Gear, 2004).

3.5.5 Sex differences in KOR (but not MOR) inhibition of latent postoperative pain

Our observations indicate that intrathecal administration of either low or high doses of CTOP equally reinstated mechanical hypersensitivity in PIM 21d male and female mice. By contrast, we found that intrathecal administration of a low dose of the KOR inhibitor LY2456302 produced a significantly more pronounced hyperalgesia and pERK activation in the dorsal horn of female mice as compared to male mice. The inability to demonstrate this sex difference with a higher dose of LY2456302 is likely due to a floor effect

associated with low mechanical thresholds. Although the sex differences following low dose LY2456302 were of small magnitude, they were statistically significant, and thus support our conclusion that tonic endogenous KOR-mediated inhibition of postoperative pain is more pronounced in females.

Consistent with our findings related to endogenous KOR inhibition of postoperative pain, clinical studies suggest a sexual dimorphism in response to exogenous pharmacological KOR agonists in the management of postoperative pain (Pande et al., 1996b; Pande et al., 1996a). For example, clinical trials using mixed-action MOR/KOR ligands and self-reported pain scales revealed that KOR agonism yields enhanced postoperative analgesia in women following third molar extraction (Gear et al., 1996a; Gear et al., 1996b, 1999). However, the literature of sex differences in the analgesic or antihyperalgesic actions of pharmacological agonists with selectivity at KOR is inconsistent or even contradictory. On one hand, KOR agonists can exert greater antinoception in male rodents (Barrett et al., 2002b; Terner et al., 2003a; Terner et al., 2003b; Sternberg et al., 2004), while others suggest that KOR agonists can exert greater antinociception in females (Mogil et al., 2003; Lomas et al., 2007; Robinson et al., 2016). Similar mixed effects have been reported in rodent models of inflammatory pain: KOR agonists can exert greater antihyperalgesia in males as compared to females (Rasakham and Liu-Chen, 2011; Auh and Ro, 2012), or greater antihyperalgesia and inhibition of spinal Fos expression in females as compared to males (Bereiter, 2001; Clemente et al., 2004; Lawson et al., 2010).

The mechanisms by which endogenous KOR modulates postoperative pain in a sex-dependent manner remains to be answered. Sex differences in KOR pharmacology

have been attributed to gonadal hormones or genetic factors (Mogil et al., 2003; Rasakham and Liu-Chen, 2011). Sex differences in KOR-mediated mechanical and heat antinociception may be mediated by estrogens (Lawson et al., 2010; Robinson et al., 2016) and progesterone (Sternberg et al., 2004). Estrogen levels regulate KOR gene expression and density (Harris and Drake, 2001; Harris et al., 2004; Kren et al., 2008; Lawson et al., 2010). Moreover, spinal estrogen signaling regulates the expression of MOR/KOR heterodimers (Liu et al., 2011), of which expression is higher in proestrus females (Chakrabarti et al., 2010). In contrast, other studies attribute the sex differences in KOR analgesia to the protective effect of androgen hormones, as testosterone negatively regulates KOR function (van Haaren et al., 2000; Rasakham and Liu-Chen, 2011; Macedo et al., 2016).

Genetics also likely contributes to sex differences in KOR analgesia. For example, the XY chromosome is positively associated with the expression of the dynorphin gene (Chen et al., 2009) and heat threshold alterations in response to KOR agonists (Gioiosa et al., 2008). In the human experimental setting, a greater response to KOR mixed-agonists on heat and ischemic pain was observed in a subset of redheaded and fair skinned women related to polymorphisms on the melanocortin-1-receptor gene (MC1R) (Mogil et al., 2003; Fillingim and Gear, 2004). Further studies are needed to determine the contribution of hormonal, neurochemical, and genetic factors to sex differences in endogenous KOR inhibition of latent postoperative analgesia.

In summary, our results indicate that surgical incision leads to long-lasting, LS of dorsal horn neurons that is tonically opposed by prolonged activation of MOR and KOR signaling in the spinal dorsal horn. We found a modest, but significant sex difference in

neuron-dependent KOR-mediated postoperative endogenous analgesia. Considering that LS represents a period of susceptibility for chronic pain, sex differences in endogenous opioid analgesia may shed some light on potential central mechanisms involved in the higher predisposition of females for persistent pain states. Indeed, failure of MOR or KOR signaling could lead to persistent postsurgical pain. A better understanding of sex-dependent endogenous pain inhibition by spinal KOR is essential for the development of new KOR-based pharmacotherapies that target pain and addiction.

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CHAPTER 4. Kappa Opioid Receptor Targets Adenylyl Cyclase 1, PKA and Epac to Maintain Latent Postoperative Pain Sensitization within a State of Remission

4.1 Abstract

We previously reported that endogenous kappa opioid receptor (KOR) activity tonically inhibits latent sensitization (LS) in a murine plantar incision model of postoperative pain for at least 3 weeks after surgical incision. We now report that intrathecal injection of the KOR selective agent, LY2456302 (CERC 501, 10µg, i.t.), restored hyperalgesia when administered much longer, 13 months, after surgical incision. We next determined whether endogenous KOR maintains latent postoperative pain by inhibiting cAMP-dependent pathways, with a focus on adenylyl cyclase isoform-1 (AC1) and its two downstream receptors, protein kinase A (PKA) and exchange protein activated by cAMP (Epac). We used AC1 knock-out (KO) mice and pharmacological inhibitors of AC1 (NB001, 1.5µg i.t.), PKA (H89, 10nmol) and $Epac_{1/2}$ (ESI-09, 10µg i.t.), and measured mechanical stimulus-evoked behaviors and immunoreactivity of phosphorylated extracellular signal-regulated kinase (pERK), a marker of LS-associated sensitization of spinal dorsal horn neurons. Postoperative hyperalgesia was attenuated in AC1 KO male mice. Moreover, AC1 gene deletion and NB001 precluded LY2456302-evoked reinstatement of hyperalgesia in both male and female mice. Furthermore, H89 prevented LY2456302-evoked reinstatement of mechanical hyperalgesia in male but not female mice, while ESI-09 reversed hyperalgesia in both sexes. We conclude that sustained KOR signaling inhibits spinal AC1-dependent mechanisms that drive postoperative LS in a sex-dependent manner.

4.2 Introduction

Persistent postoperative pain develops in millions of patients that undergo a variety of surgical procedures (Kehlet et al., 2006). Opioids remain the mainstay analgesics of acute postoperative pain; however, prolonged opioid therapy may lead to life-threating adverse effects, tolerance, dependence, addiction liability, and paradoxical pain exacerbation. A better understanding of the cellular mechanisms that drive persistent pain states, and opposing endogenous analgesia provided by opioid receptor signaling, may lead to the development of novel treatments for chronic postoperative pain. To this end, we employed a murine model of chronic postoperative pain that involves plantar incision of the hindpaw, followed by a waiting period of multiple weeks. Although hyperalgesia has resolved, what remains is a silent, long-lasting sensitization of nociceptive neurons in the dorsal horn (Solway et al., 2011; Taylor and Corder, 2014). This "latent sensitization (LS)" is masked by compensatory activity of multiple pain-inhibitory G-protein coupled receptors including μ-opioid receptor constitutive activity (MOR_{CA} (Corder et al., 2013), the kappa opioid receptor (KOR) [Custodio et al, submitted], and the neuropeptide Y (NPY) Y1 receptor (Fu et al., 2019). We previously reported that MOR_{CA} and Y1R suppress an LS that is driven by an NMDA receptor → adenylyl cyclase-1 (AC1) signaling mechanism after chronic inflammation (Corder et al., 2013; Fu et al., 2019).

For several reasons, we hypothesized that AC1 may also mediate the LS that is masked by KOR. First, KOR is an inhibitory G- protein-coupled receptor (GPCR), and as such inhibits the activity of the adenylyl cyclases (AC) (Childers et al., 1992). Adenylyl cyclase 1 (AC1) is a Ca^{+2} - activated protein that acts as a key modulator for the induction of pathological pain in the CNS (Wu et al., 1995; Liauw et al., 2005; Wei et al., 2006;

Zhuo, 2012). It couples the intracellular Ca^{+2} derived from NMDAR activation to downstream activation of an array of downstream proteins and kinases (Zhuo, 2012). Similar to MOR, persistent activation of KOR by an exogenous drug can also lead to an AC "overshoot" (Avidor-Reiss et al., 1997; Nakagawa et al., 1999).

AC1 – cAMP signaling activates multiple downstream targets, including the canonical protein kinase A (PKA) and an alternative pathway via Epac protein family (Cheng et al., 2008). Epacs (Epac, isoform 1 and Epac, isoform 2) act as guanine nucleotide exchange factors for small GTPases (de Rooij et al., 1998; Bos, 2003), activating downstream PKC dependent pathways (Griffin, 2005; Hucho et al., 2005). Both PKA and Epac isoforms have been implicated in the development of persistent hyperalgesia after inflammation or tissue injury (Wang et al., 2013; Gu et al., 2016; Singhmar et al., 2016; Matsuda et al., 2017; Singhmar et al., 2018).

The current studies were designed to test the hypothesis that surgical incision establishes a long-lasting activation of KOR signaling that masks LS, in part through tonic inhibition of pronociceptive AC1 signaling to downstream cAMP target proteins: protein kinase A (PKA) and/or exchange protein activated by cAMP (Epac). To address this hypothesis, we performed plantar incision in male and female mice, waited for behavioral signs of hyperalgesia to resolve (21 days), and targeted spinal KOR, AC1, and cAMP receptors with the intrathecal delivery of pharmacological antagonists (and in the case of AC1, we also used AC1-/- mice), and then evaluated behavioral signs of hyperalgesia and spinal nociceptive sensitization. The latter was assessed by the immunohistochemical expression of the phosphorylated extracellular-signaling kinase (pERK) in the dorsal horn.

4.3 Materials and Methods

4.3.1 Animals

Male and female mice, 8-12 week of age were obtained from commercial sources (C57Bl/6, Charles Rivers Laboratories) or bred in-house (adcy1$^{-/-}$ = AC1 knockout and wild-type littermate AC1$^{+/+}$ controls, originally provided as a gift from Dan Storm, University of Washington). Genotype was confirmed by tail-snip PCR before the beginning of the studies. Animals were housed in a temperature-controlled room on a 14:10-hr light/dark cycle and were provided food and water *ad libitum*. All animal use protocols were approved by the IACUC at the University of Kentucky. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky protocol (#2017-2768), in accordance with American Veterinary Medical Association guidelines. Efforts were made to reduce the number of animals and their suffering. Mice were acclimated to the colony housing room for at least 4 days, and then handled for 4 days for 5 minutes prior to the initiation of the experiments.

4.3.2 Plantar Incision Model of Postoperative Pain (PIM)

Post-operative pain was induced by longitudinal incision of the plantaris muscle, as previously described (Pogatzki and Raja, 2003; Jang et al., 2011). Under isoflurane anesthesia (5% induction and 1.5-2% maintenance, via a nose cone) and antisepsis of the left hind paw with Chlorascrub® then alcohol, a #11 scalpel blade was used to perform a 5mm through the skin and fascia using, beginning 2mm from the proximal edge of the heel extending towards the digits. The underlying muscle was raised with a curved forceps, extended 4 mm and incised longitudinally, leaving the origin and insertion of the muscle intact. The overlying skin was closed with synthetic 5-0 sutures (PDS*II, Ethicon),

followed by application of antibiotic ointment was applied. Surgery was typically completed within 5-10min. Sutures were removed on the post-operative day 10. Sham controls received anesthesia but no surgical incision.

4.3.3 Direct Intrathecal (i.t.) Drug Delivery

As previously described (Fairbanks, 2003), non-anaesthetized mice were lightly restrained in a towel, and a 30G x1/2 needle (Becton Dickinson) attached to a 25-ul Hamilton micro syringe, was inserted into the subarachnoid space between the L5/L6 vertebrae. The needle was angled about 30-45 degrees with the horizontal plane and slightly advanced until reflexive tail flick was confirmed, then 5 µl of drug or vehicle was slowly administered. The needle was held in place for 30sec, to ensure the solution fully enters the spinal canal, withdrawn, and then the mouse was returned to its testing chamber.

4.3.4 Drug Dosing

We administered 5ul of the following drugs via intrathecal route at 21 postoperative day mice: the compound LY2456302 (gift from NIDA Drug Supply Program) 10µg (Rorick-Kehn et al., 2014a; Rorick-Kehn et al., 2014b) was dissolved in ddH20 and sonicated at 30°C for 30minutes until complete homogenization of the drug was achieved. LY2456302 (Med Chem Express) 10µg was dissolved in 1:1:8 (EtOH / Chremophor (Sigma-Aldrich) / Saline) and injected at a dose of 10µg. Other drugs dissolved in saline were NB001 1.5µg (Tocris); 6Bnz-cAMP 10nmol (BioLog); H89 10nmol (Tocris); 8-CPT 3nmol (BioLog). ESI-09 10µg (Almahariq et al., 2013; Chen et al., 2013) (gift from Jia Zhuo, University of Texas Medical Branch) was dissolved in 1:1:8 (EtOH / Chremophor / Saline). Dose-response studies previously performed in our laboratory informed our dose of LY2456302 (Custodio et al, submitted), NB001 were determined based on (Corder et

al., 2013), 6Bnz-cAMP, H89, 8-CPT, and ESI-09 (Fu et al., 2019). Control animals for the behavioral assays received 5µl (i.t.) of the vehicle (sterile saline or 1:1:8 (EtOH / Chremophor / Saline). All compounds were injected i.t. at least 3 weeks post-operatively, after mechanical thresholds had returned to baseline levels. Some animals from figures 4.1A and 4.1C, 4.2A-D, 4.5A-D, and 4.7A-D were tested in a cross-over design receiving saline or drug a week after the first inthratecal injection. Animals were matched to experimental groups by a different investigator.

4.3.5 Mechanical Hyperalgesia Testing

All animals were acclimated in a temperature and light controlled room within individual Plexiglas boxes placed on the top of a stainless-steel mesh platform for 30 to 60 min before behavioral testing. Mechanical hypersensitivity thresholds were assessed using an incremental series of 8 von Frey filaments (Stoelting, Illinois) of logarithmic stiffness (approximately 0.008-6 grams). The 50% withdrawal threshold was determined using an up-down method (Chaplan et al., 1994). Each filament was applied perpendicular to the surface of the skin just lateral to the incision site with enough force to cause a slight bending of the filament. A positive response was defined by a rapid withdrawal of the paw within 5 seconds. Using the up-down method (Chaplan et al., 1994), the gram forces were logarithmically converted into 50% of mechanical thresholds. This test was performed by an observer blind to treatment at baseline up to 21 days post-PIM to confirm the development of mechanical allodynia and assess the reinstatement of hyperalgesia three weeks after surgery. Animals were assigned to experimental groups in all studies by a different investigator than the examiner that remained blinded until the end of all experiments.

4.3.6 Phosphorylated Extracellular Signal-Regulated Kinase (pERK) Stimulation

At 21 days after surgery mice received either vehicle or LY2456302 (10µg). Two hours after injection, mice were lightly anesthetized with isoflurane (1.5%), and the ventral surface of the ipsilateral hindpaw was mechanically stimulated with gentle 2-second stroke with a cotton swab from heel to toe. This was repeated every 5s for 5min. After an additional 5 min pause, mice were deeply anesthetized with isoflurane and transcardially perfused with cold 0.01M phosphate buffer saline (PBS, Fischer Scientific) /heparin buffer (10,000 USP units/L), followed by 10% phosphate formalin buffer. Lumbar spinal cords were harvested and post-fixed in the same fixative overnight at 4ºC, and then cryoprotected with 30%sucrose until total submersion (1-3 days).

4.3.7 Histology and Immunohistochemistry

Transverse sections (30µm) from L4 were obtained on a sliding microtome (Leica, SM 2000R). A series of non-consecutive sections was washed in 0.01M PBS, blocked in 3% normal serum (goat or donkey; Gemini Bioproducts), containing 0.3% Triton X-100 (Sigma Aldrich) in 0.01M PBS for 1h, and then incubated with primary rabbit antibody anti-phosphorylated-ERK ½ antiserum (1:800, Cell Signaling) at 4ºC for 36 hours on a shaker. Sections were washed in 0.01M PBS and incubated with secondary goat anti-rabbit Alexa Fluor 568 conjugated antibody (1:1000, Invitrogen) for 1h. The sections were washed in 0.01M phosphate buffer, mounted and cover slipped with mounting medium with DAPI (Vectashield, Vector laboratory).

Five good quality sections from spinal cord segments L4 per animal, in part were randomly selected from each subject for pERK analyses. The number of pERK-positive

cells on Rexed laminae I and II, and III-V were counted by a blinded examiner. All images were captured with a Nikon TE-2000 microscope equipped with NIS-Elements AR 4.13.05 (Nikon), and the analyses performed with NIS-Elements Software AR analysis 4.13.05 Software offline (Nikon).

4.3.8 Statistical Analysis

Data was entered into Prism software (version 8.0, GraphPad, La Jolla, CA). All data are presented as mean values with the corresponding standard error (mean ± SEM). Two-way ANOVA repeated measures (RM) was generated to analyze differences in means among groups over time. Drugs and doses were group factors and time was a repeated measure. If a significant interaction was found, ANOVA was followed by Bonferroni post-hoc analyses. Three-way ANOVA repeated measures was used to determine effects of sex and treatment overtime as appropriate. Area under the curve (AUC) or integrated thresholds was plotted using the trapezoidal method and analyzed using ordinary two-way ANOVA followed by Bonferroni. Statistical significance was set at $p<0.05$ (**Tables 4.1 to 4.3**).

4.3.9 Experimental blinding procedures

All the behavioral and immunofluorescence assays were conducted by the same investigator (Lilian Custodio) who was blinded to the experimental groups and drugs during all experiments. All the drugs and vehicles were prepared and disposed into identical tubes and volume. The animals were allocated to the experimental groups by a different examiner (Diogo dos Santos, Renee Donahue or Liping Zhang) who performed the tube coding using a letter system. For the Epac and PKA studies due to the different appearance (color) of the drugs and vehicles used that prevents an entirely blind procedure,

the animals were allocated in the experimental groups and injected by a different examiner (Diogo dos Santos or Renee Donahue after being acclimated to the animals). After the completion of the experiment and data plotting in an excel sheet, the code was handled to the examiner and the data analyzed. For the pERK experiments, to ensure blinding, the animals were randomly handled to the experimenter by a colleague for injections and paw stimulation. After completion of the experiment, the investigator was unblinded for the groups.

Table 4.1 Statistics for Figures 4.1 and 4.2.

Measure	Figure	RM Two-way ANOVA
Mechanical Thresholds	Figure 4.1A (males)	Drug: $F_{1,10} = 23.23$, $p=0.0007$ Time: $F_{4.87,48.72} = 22.13$, $p=0.001$ Drug vs Time: $F_{11,110} = 9.40$, $p<0.0001$
	Figure 4.1B (males)	Drug: $F_{1,13} = 22.60$, $p=0.0004$ Time: $F_{11,143} = 23.38$, $p<0.0001$ Drug vs Time: $F_{11,143} = 5.72$, $p<0.0001$
	Figure 4.1C (males)	Drug: $F_{1,13} = 6.41$, $p=0.02$ Time: $F_{11,143} = 31.69$ $p<0.0001$ Drug vs Time: $F_{11,143}= 3.54$, $p=0.0002$
	Figure 4.2A (males and females)	Genotype: $F_{1,33} = 8.15$, $p=0.007$ Time: $F_{3.31,109.4} = 178.2$, $p<0.0001$ Genotype vs Time: $F_{5,165} = 3.29$, $p=0.007$
	Figure 4.2B (males)	Genotype: $F_{1,16} = 4.77$, $p=0.04$ Time: $F_{3.29,52.77} = 93.56$, $p<0.0001$ Genotype vs Time: $F_{5,80} = 3.69$, $p=0.004$
	Figure 4.2C (females)	Genotype: $F_{1,15} = 3.24$, $p=0.09$ Time: $F_{2.56, 38.51} = 81.56$, $p<0.0001$ Genotype vs Time: $F_{5,75} = 0.83$, $p=0.52$

Table 4.2 Statistics for Figures 4.2 to 4.8

Measures	**RM Two-way ANOVA**					
	Males and Females		**Males**		**Females**	
Mechanical Thresholds	Figure 2A	Group: $F_{3,52} = 14.76$ $p<0.0001$ Time: $F_{3.80,197.8}=$ 18.82 $p<0.0001$ Group x Time: $F_{27,46}=$ 6.25 $p<0.0001$	Figure 2B	Group: $F_{3,24} =5.08$ $p=0.007$ Time: $F_{2.56,61.62}=$ 8.68 $p=0.0002$ Group x Time: $F_{27,216}=$ 4.15 $p<0.0001$	Figure 2C	Group: $F_{3,24} =10.38$ $p=0.0001$ Time: $F_{4.76,114.4}=$ 10.51 $p<0.0001$ Group x Time: $F_{27,216}=$ 3.12 $p<0.0001$
	Figure 3A	Group: $F_{3,27}=3.44$ $p=0.03$ Time: $F_{3.64,98.36}=$ 91.44 $p<0.0001$ Group x Time: $F_{18,162} =$ 5.00 $p<0.001$	–		–	

Table 4.2 Statistics for Figures 4.2 to 4.8 (continued).

Mechanical Thresholds	Figure 5A	Group: $F_{2,52}$= 4.44 p=0.01 Time: $F_{2.76,142.8}$= 19.86 p<0.0001 Group x Time: $F_{6,156}$= 3.93 p=0.001	Figure 5B	Group: $F_{2,24}$ =5.24 p=0.01 Time: $F_{2.77,66.62}$= 13.78 p<0.0001 Group x Time: $F_{6,72}$= 3. 58 p=0.003	Figure 5C	Group: $F_{2,25}$ =0.58 p=0.56 Time: $F_{2.61,65.36}$= 5.94 p<0.0001 Group x Time: $F_{6,75}$= 1.03 p=0.41
	Figure 6A	Group: $F_{3,44}$= 10.51 p<0.0001 Time: $F_{6,264}$= 169.3 p<0.0001 Group x Time: $F_{18,264}$= 6.73 p<0.0001	Figure 6B	Group: $F_{3,20}$= 4.37 p<0.0001 Time: $F_{6,120}$= 86.10 p<0.0001 Group x Time: $F_{18,120}$= 2.76 p=0.0005	Figure 6C	Group: $F_{3,20}$ = 7.29 p=0.001 Time: $F_{6,120}$= 95.05 p<0.0001 Group x Time: $F_{18,120}$= 4.99 p<0.0001

Table 4.2 Statistics for Figures 4.2 to 4.8 (continued).

Mechanical Thresholds	Figure 7A	Group: $F_{2,33}$= 6.56 p=0.004 Time: $F_{2.83,93.45}$= 19.61 p<0.0001 Group x Time: $F_{6,99}$= 5.27 p<0.0001	Figure 7B	Group: $F_{2,18}$ =4.85 p=0.02 Time: $F_{2.29,41.39}$= 10.45 p<0.0001 Group x Time: $F_{6,54}$= 3.01 p=0.01	Figure 7C	Group: $F_{2,15}$ =2.95 p=0.08 Time: $F_{2.49,37.36}$= 9.13 p=0.0003 Group x Time: $F_{6,45}$= 3.60 p=0.005
	Figure 8A	Group: $F_{3,44}$= 4.53 p=0.007 Time: $F_{6,264}$= 154.0 p<0.0001 Group x Time: $F_{18,264}$= 5.23 p<0.0001	Figure 8B	Group: $F_{3,20}$= 2.98 p=0.05 Time: $F_{6,120}$= 94.45 p<0.0001 Group x Time: $F_{18,120}$= 3.99 p=0.0005	Figure 8C	Group: $F_{3,20}$ = 2.13 p=0.12 Time: $F_{6,120}$= 63.67 p<0.0001 Group x Time: $F_{18,120}$= 2.21 p=0.005

Table 4.3 Area under the curve (AUC) analyses

Measure	Figure	Area Under the Curve (AUC)
Mechanical Thresholds	Figure 4.2D (males and females)	Sex: $F_{1,48} = 0.01$, $p=0.89$ Drug: $F_{3,48} = 26.34$, $p<0.0001$ Sex x Drug: $F_{3,48} = 0.29$, $p=0.82$
	Figure 4.3B (males and females)	Sex: $F_{1,12} = 1.06$, $p=0.32$ Drug: $F_{1,12} = 16.59$, $p<0.0001$ Sex x Drug: $F_{3,48} = 0.05$, $p=0.82$
	Figure 4.5D (males and females)	Sex: $F_{1,33} = 5.30$, $p=0.02$ Drug: $F_{1,33} = 9.29$, $p=0.004$ Sex x Drug: $F_{1,33} = 2.59$, $p=0.11$
	Figure 4.6D (males and females)	Sex: $F_{3,40} = 1.14$, $p=0.29$ Group (Drug): $F_{1,40} = 15.62$, $p<0.0001$ Sex x Group: $F_{3,40} = 0.95$, $p=0.42$
	Figure 4.7D (males and females)	Sex: $F_{1,20} = 2.13$, $p=0.15$ Drug: $F_{1,20} = 25.31$, $p<0.0001$ Sex x Drug: $F_{1,20} = 0.28$, $p=0.59$
	Figure 4.8D (males and females)	Sex: $F_{1,40} = 1.58$, $p=0.21$ Group: $F_{1,40} = 8.78$, $p=0.0001$ Sex x Group: $F_{3,40} = 1.66$ $p=0.18$

Table 4.3 Area under the curve (AUC) analyses (continued)

#pERK +cells	Figure 4.4I (males and females) Lam I-V	Genotype: $F_{1,28} = 16.46$, $p=0.0004$ Region: $F_{1,28} = 35.35$, $p<0.0001$ Genotype vs Region: $F_{1,28} = 14.35$, $p=0.0007$
	Figure 4.4J (males and females) Lam I-II, III-V	Genotype: $F_{1,56} = 25.23$, $p<0.0001$ Region: $F_{3,56} = 37.86$, $p<0.0001$ Genotype vs Region: $F_{3,56} = 11.10$, $p<0.0001$
	Figure 4.4K (males vs. females) Lam I-V	Genotype: $F_{1,12} = 16.33$, $p=0.001$ Group (Sex vs region): $F_{1,12} = 0.48$, $p=0.50$ Genotype vs Group: $F_{1,12} = 0.75$, $p=0.40$
	Figure 4.4L (males and females) Lam I-II, III-V	Region: $F_{1,24} = 26.11$, $p<0.0001$ Group (Sex vs genotype): $F_{3,24} = 7.26$, $p=0.001$ Region vs Group: $F_{3,24} = 2.20$, $p=0.11$

Table 4.4 Three-way ANOVA analyses

Measure	RM Three-way ANOVA (treatment x sex x drug)	
Mechanical Thresholds	Figures 5B-C (males and females)	Drug: $F_{1,33} = 4.42$, $p=0.04$ Time: $F_{2.563, 84.59} = 19.17$, $p<0.0001$ Sex: $F_{1,33} = 4.47$, $p=0.04$ Drug vs Time: $F_{3,99} = 4.53$, $p=0.005$ Drug vs Sex: $F_{1,33} = 2.01$, $p=0.16$ Time vs Sex: $F_{5,165} = 1.36$, $p=0.24$ Time vs Sex vs Drug: $F_{3,99} = 0.69$, $p=0.55$
	Figure 7B-C (males and females)	Drug: $F_{1,20} = 7.84$, $p=0.01$ Time: $F_{2.65,52.99} = 29.82$, $p<0.0001$ Sex: $F_{1,20} = 1.80$, $p=0.19$ Drug vs Time: $F_{3,60} = 10.81$, $p<0.0001$ Drug vs Sex: $F_{1,20} = 0.16$, $p=0.69$ Time vs Sex: $F_{3,60} = 0.41$, $p=0.74$ Time vs Sex vs Drug: $F_{3,60} = 1.43$, $p=0.24$

4.4 Rationale and Results

4.4.1 Kappa-opioid receptors signal inhibit LS for at least 1 year after surgery

Our laboratory demonstrated that repeated intrathecal (i.t.) and systemic delivery of the inverse agonist / non-selective opioid receptor antagonist, naltrexone, reinstated hyperalgesia when administered a few months after intraplantar complete Freund's adjuvant (CFA) administration (Corder et al., 2013). Ongoing studies also indicates that naltrexone reinstates mechanical hypersensitivity up to 1 year after plantar incision (dos Santos, unpublished observations). As discussed at Chapter 3, mechanical hypersensitivity persists up to 14 to 21 days after surgical incision of the hindpaw. We demonstrated that MOR and KOR are the opioid receptor systems involved in the masking of LS. To determine whether tonic KOR signaling also persists for such a long time beyond tissue healing, we performed the plantar incision model (PIM), waited for the resolution of mechanical hypersensitivity, and then intrathecally administered a KOR selective antagonist (LY2456302) 21 days, 6 months and 13 months after surgery.

As illustrated in **Figure 4.1**, we found that LY2456302, but not vehicle, reinstated mechanical hyperalgesia when administered at either 21 days $F(11, 110) = 9.40$, $p=0.0001$), 6 months $F(11, 143) = 5.72$, $p<0.0001$) or 13 months $F(11, 143) = 3.54$, $p=0.0002$, **Table 4.1**) after surgery. Our findings indicate that LS and opposing KOR signaling persist for over a year after surgery.

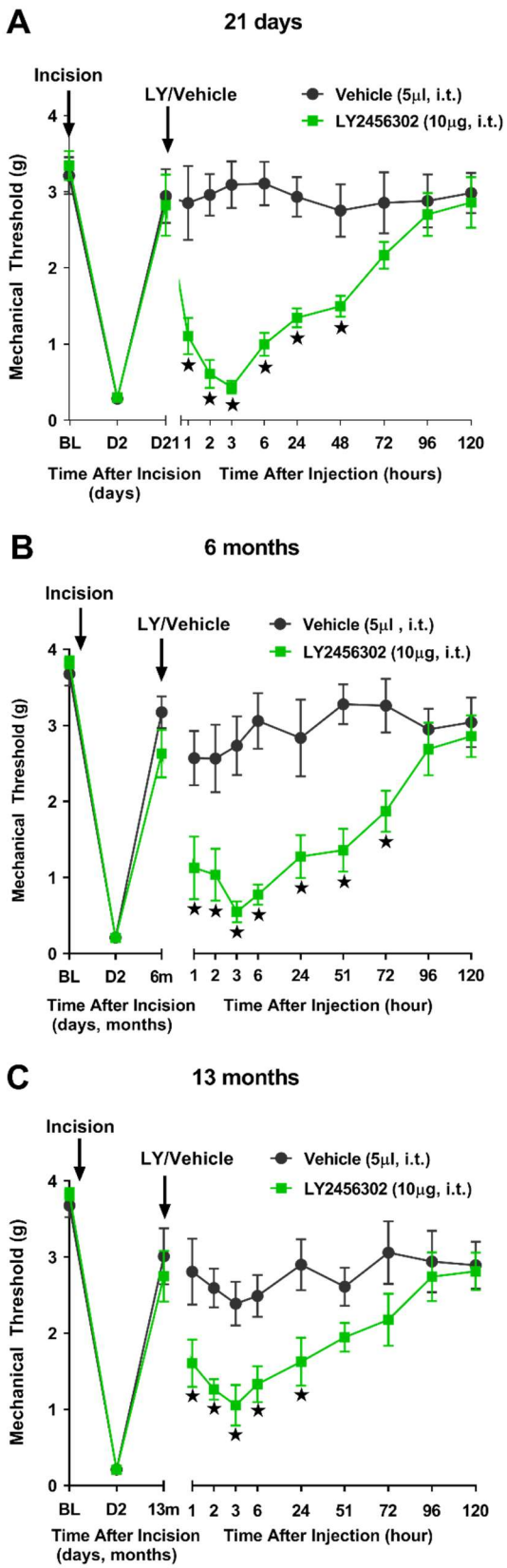
A
21 days
Incision
LY/Vehicle
Vehicle (5μl, i.t.)
LY2456302 (10μg, i.t.)
Mechanical Threshold (g)
0
1
2
3
4
BL
D2
D21
1
2
3
6
24
48
72
96
120
Time After Incision (days)
Time After Injection (hours)
B
6 months
Incision
LY/Vehicle
Vehicle (5μl , i.t.)
LY2456302 (10μg, i.t.)
Mechanical Threshold (g)
0
1
2
3
4
BL
D2
6m
1
2
3
6
24
51
72
96
120
Time After Incision (days, months)
Time After Injection (hour)
C
13 months
Incision
LY/Vehicle
Vehicle (5μl, i.t.)
LY2456302 (10μg, i.t.)
Mechanical Threshold (g)
0
1
2
3
4
BL
D2
13m
1
2
3
6
24
51
72
96
120
Time After Incision (days, months)
Time After Injection (hour)

Figure 4.1. Endogenous kappa opioid inhibition of LS persists at least 13 months after surgical incision.

Mechanical thresholds at baseline (BL), 2 days, and **(A)** 21 days, **(B)** 6 months, **(C)** up to 13 months after plantar incision in male C57Bl/6 mice. Intrathecal administration of selective KOR antagonist LY2456302 (10µg), reinstated mechanical hypersensitivity when given (A) 21 days, (B) 6 months, (C) up to 13 months after PIM (n=6-8). BL = baseline behavior prior to drug administration. Data represented as mean +/- SEM. ★ $p<0.05$. Bonferroni's post-hoc tests following repeated measures of 2-way ANOVA.

4.4.2 Prolonged kappa opioid receptor signaling silences behavioral and neuronal signs of postoperative LS via inhibition of adenylyl cyclase, isoform 1 (AC1)

We investigated whether prolonged KOR signaling opposes LS via inhibition of AC1. To answer this question, we used two approaches, an AC1 inhibitor, and $AC1^{-/-}$ mutant mice. Genetic and pharmacological interruption of AC1 does not change transient nociception in uninjured animals (Wei et al., 2002), but prevents CFA-induced hyperalgesia (Wei et al., 2002; Wang et al., 2011; Corder et al., 2013), and latent sensitization (Corder et al., 2013; Fu et al., 2019). To test the hypothesis that KOR inhibits AC1 to maintain latent postoperative pain sensitization in remission, we evaluated LY2456302 (10µg)-induced reinstatement of hyperalgesia in AC1 knock out mice and after inthratecal (i.t.) administration of the AC1 inhibitor, tested 21 days after plantar incision.

As illustrated in **Figure 4.2**, AC1 KO mice and their wild-type controls exhibited similar baseline mechanical thresholds, regardless of sex. After incision, the time course of hyperalgesia differs across strain and sex. In males, post-incisional hyperalgesia in AC1 knockouts resolved faster than wild-type mice ($F (5, 165) = 3.29$; $p=0.007$, Table 4.1, Figure 4.2A). However, when stratified by sex, AC1 KO accelerates the recovery of hyperalgesia in male ($F (5, 80) = 3.69$; $p=0.004$, Table 4.1, Figure 4.2B), but not female mice ($F (5, 75) = 0.83$; $p=0.52$, **Table 4.1**, Figure 4.2C). Moreover, AC1 gene deletion abolished reinstatement of hyperalgesia induced by KOR antagonist in wild-type animals ($F (27, 468) = 6.25$; $p<0.0001$, Figure 4.2A) of either sex ($F (27, 216) = 4.15$; $p<0.0001$ for males, fig. 4.2B; $F (27, 216) = 3.12$; $p<0.0001$ for females, **Table 4.2,** Figure 4.2C).

Integrated analyses over the 1-48h timepoints further illustrates that LY2456302-induced reinstatement of hyperalgesia is different between the strains (drug vs genotype, $F(3, 48) = 26.34$, $p < 0.0001$, **Table 4.3**, Figure 4.2D).

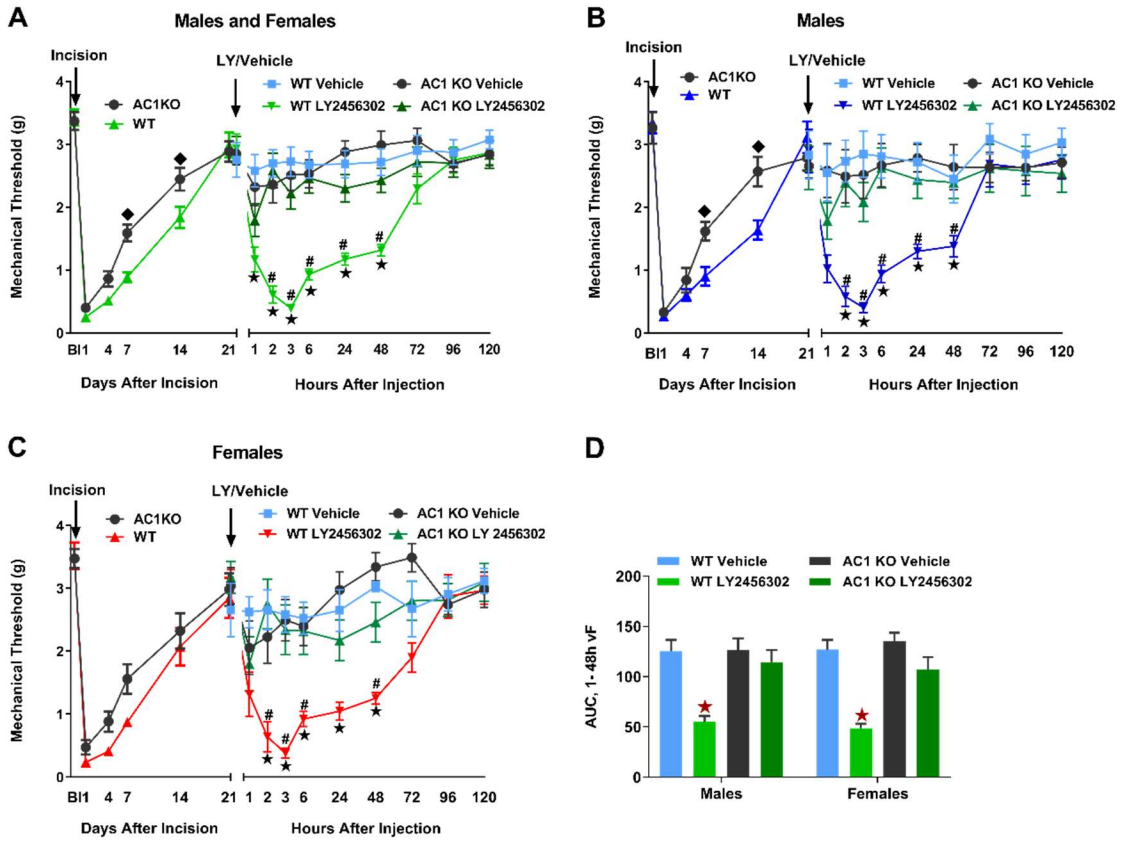

Figure 4.2. AC-1 gene deletion precludes KOR antagonist-induced postoperative hyperalgesia.

(**A-C**) Mechanical thresholds at baseline (BL), 1, 4, 7, and 21 days after plantar incision in C57Bl/6 and AC1 KO. Gene deletion of AC1 accelerates the recovery of mechanical thresholds after surgery in male (**B**)**,** but not female mice (**C**). AC1 gene deletion prevented LY2456302 (10µg, i.t.) – induced hyperalgesia in both male and female mice (**A - D**). The area under the curve (AUC, 1-48h) further illustrates the effect of AC1 gene deletion on LY2456302- (10 µg) induced reinstatement of mechanical hypersensitivity in the ipsilateral hindpaw (**D**). (n = 6-15 per group) ◆ $p < 0.05$ WT compared to $AC1^{-/-}$ mutant. ★ $p < 0.05$ LY2456302 compared to vehicle. # $p < 0.05$ WT LY2456302 compared to AC1 LY2456302. ★ $p<0.05$ WT LY compared to WT vehicle, AC1 vehicle and AC1

LY2456302. Bonferroni's post-hoc tests followed repeated measures of 2-way ANOVA. Data represented as mean +/- SEM.

Next, we used a selective inhibitor of AC1, NB001 (1.5µg, i.t.) (Wang et al., 2011; Corder et al., 2013). We performed plantar incision and assessed mechanical hypersensitivity two days later. In 21d post-incisional mice (PIM) mice, we pre-administered NB001 15 minutes before LY2456302 (10µg, i.t.). Consistent with our previous studies, spinal delivery of LY2456302 elicited reinstatement of mechanical hypersensitivity at 2h and 3h time points ($p=0.003$ and $p=0.001$ respectively, **Figure 4.3A**). In agreement with the AC1 KO study, NB001 abolished the reinstatement precipitated by LY2456302 ($F (15, 135) = 4.00$; $p<0.0001$, Figure 4.3A). This further illustrated by integrated analysis over 1-2h timepoints after the drug administration (drug effect, $F (1, 6) = 21.89$; $p = 0.003$, Table 4.3, **Figure 4.3B**).

To test the hypothesis that AC1 contributes to latent sensitization of dorsal horn neurons, we evaluated the effect of AC1 deletion on a measure of neuronal activation in the dorsal horn, phosphorylated extracellular signal-regulated kinase (pERK). We administered LY2456302 (10 µg) intrathecally, and 2h later, at the peak of hyperalgesia, we performed light touch stimulation in the hindpaw of WT and AC1-/- mice. We found that the number of stimulus-evoked pERK+ profiles was significantly reduced in AC1 KO as compared to WT mice in the ipsilateral dorsal horn (laminae I-V) ($F (1, 28) = 14.35$, $p=0.0007$, **Figure 4.4I**) in both superficial (laminae I-II) and deep dorsal horn (laminae III-V) ($F (1, 56) = 11.10$, $p<0.0001$, **Figure 4.4J**). As illustrated in **Figure 4.4K – L**, AC1 deletion decrease of nociceptive activation occurs in the ipsilateral dorsal horn laminae I-V of females ($F (1, 12) = 16.33$, $p<0.0001$, fig. 4.4.K) and superficial dorsal horn of either sex (effect of group $F (3, 24) = 7.26$, $p=0.01$, Table 4.3, Figure 4.4.L).

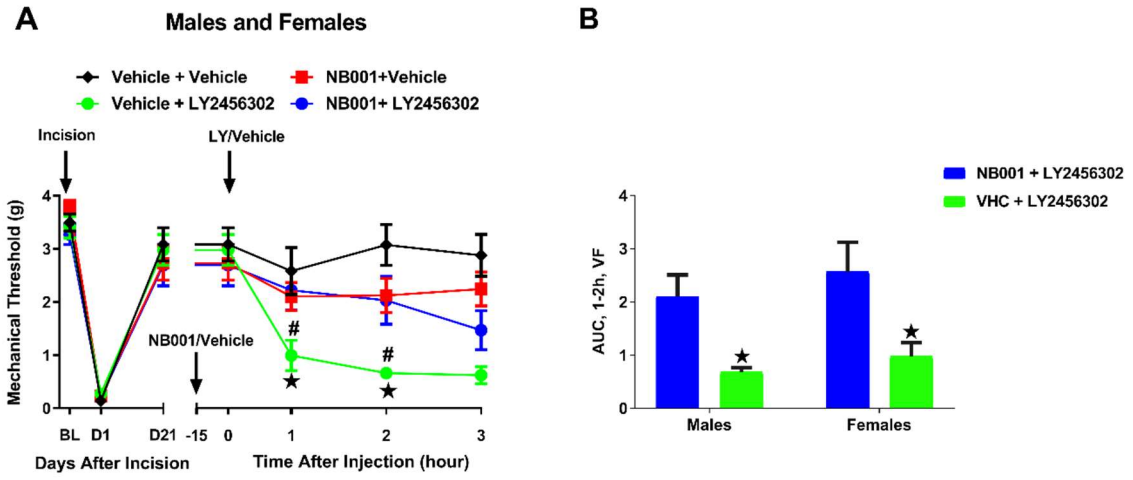

Figure 4.3. Pharmacological inhibition of AC1 prevented LY2456302-induced reinstatement of postoperative hyperalgesia.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male C57Bl/6 mice. (**A**) Pre-administration of the AC1 inhibitor NB001 (1.5µg, i.t.) prevented LY2456302 (10µg) – induced reinstatement of hyperalgesia in both male and female mice. (**B**) The area under the curve (AUC) further illustrates that LY induced reinstatement in both sexes (n= 3-8 per group). ★ $p < 0.05$ LY compared to vehicle. # $p < 0.05$ NB001 + LY2456302 compared to Vehicle + LY2456302. BL = baseline behavior prior to drug administration. Bonferroni's post-hoc tests following repeated measures of 2-way ANOVA. Data represented as mean +/- SEM.

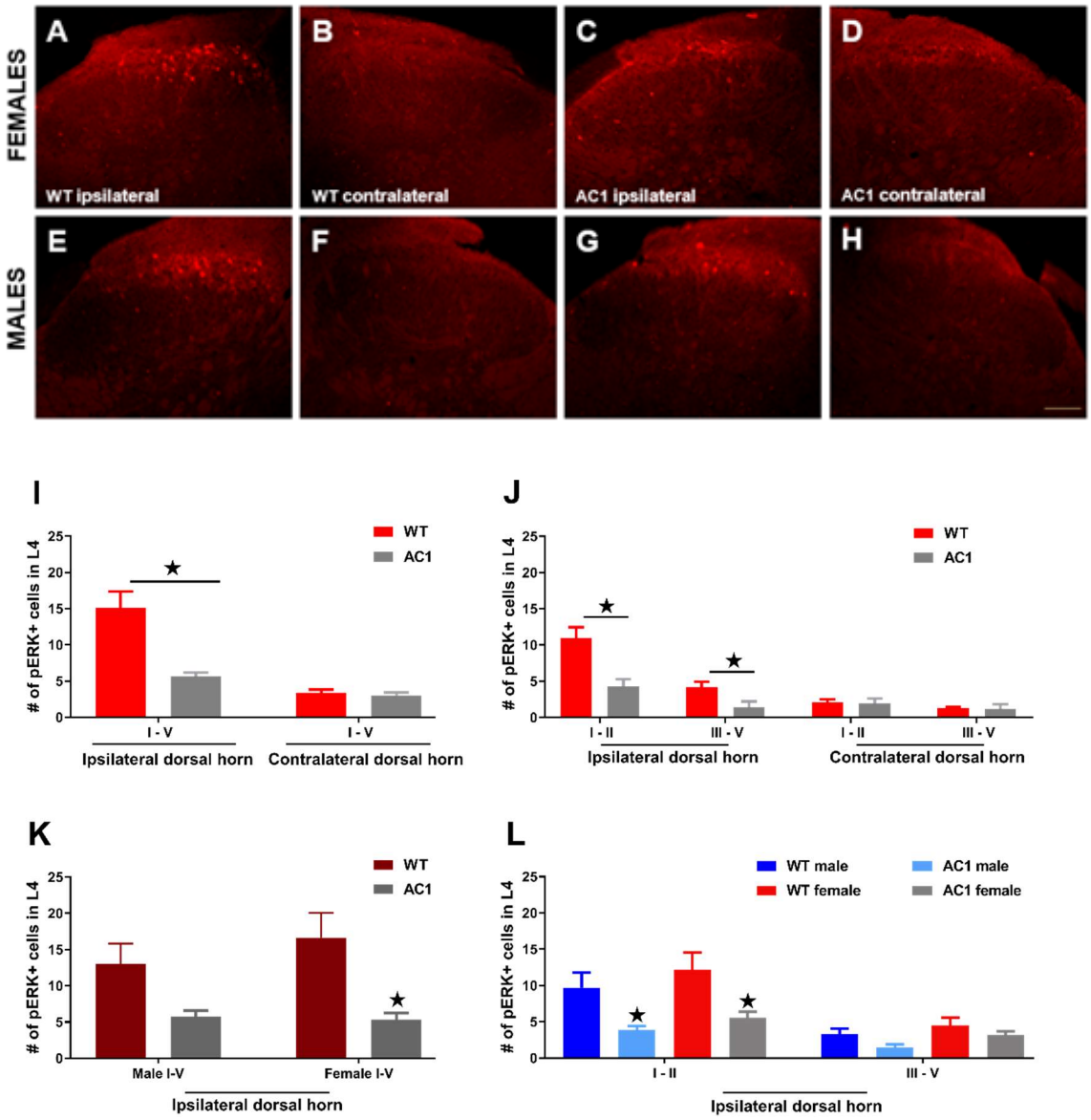

Figure 4.4. AC1 gene deletion prevented LY2456302 - induced reinstatement of neuronal activity in the dorsal horn.

Representative images of pERK immunoreactivity in the dorsal horn of female (**A-D**), and male (**E-H**) mice. (A, E) LY2456302 (10 µg, i.t.) enhanced the number of pERK+ cell profiles in WT mice in the ipsilateral side (A, E) as compared to the contralateral side (B, F). In contrast to WT, few pERK-positive cells were observed in AC1-/- mice ipsilateral

paw (C, G) compared to contralateral (D, H). (**I - J**) Histograms illustrate that LY2456302 i.t. administration increased the number of pERK+ cells in WT compared to AC1-/- male and female mice. Images were taken with a 10X objective. n = 8 per group/4 per sex. Data represent mean +/- SEM. ★ $p<0.05$. Bonferroni's post-hoc tests following 2-way ANOVA. Scale bars: 100μm.

4.4.3 PKA activation reinstates behavioral signs of postoperative hyperalgesia in male but not female mice

Recent evidence from our laboratory suggests that the cAMP receptors, PKA and Epac1 and Epac2, are activated after nerve injury and intraplantar CFA in male rodents (Fu et al., 2019). Our findings indicate that LS is driven by pronociceptive AC1 signaling in the dorsal horn in both male and female mice. Therefore, we hypothesized that AC1 signaling recruits both PKA and Epac downstream signaling.

We recently reported that intrathecal administration of the selective cAMP analog 6-bnz-cAMP (6-bnz, 10nmol) reinstated mechanical when tested in male mice that received intraplantar CFA 21 days earlier (Fu et al., 2019), but this study was restricted to male mice. As illustrated in **Figure 4.5A**, 6-bnz reinstated hyperalgesia in 21d PIM mice ($F(6,156) = 3.93$, $p=0.001$). The 3-way ANOVA analyses revealed significant effects of sex, drug and time ($p=0.04$, $p=0.04$ and $p<0.0001$ respectively, **Table 4.4**). When the data were stratified by sex, we found that 6-bnz reinstated mechanical hypersensitivity 21d PIM male mice (RM Two-way ANOVA, drug effect $F(2, 24) = 8.84$, $p=0.01$ and drug and time interaction $F(6, 72) = 3.58$, $p=0.003$, Table 4.2, **Figure 4.5B**), and showed no effect in female mice (RM Two-way ANOVA, drug effect $F(2, 25) = 0.58$, $p=0.56$ and drug and time interaction $F(6, 75) = 1.03$, $p=0.041$, Table 4.2, **Figure 4.5C**). As illustrated in **Figure 4.5D** integrated analysis over time revealed the effects of sex ($F(1, 33) = 5.30$, $p=0.02$) and drug ($F(1, 33) = 9.29$, $p=0.004$, Table 4.3) on 6-bnz induced-reinstatement of mechanical hypersensitivity ($p=0.01$ and $p>0.99$ for males and females respectively).

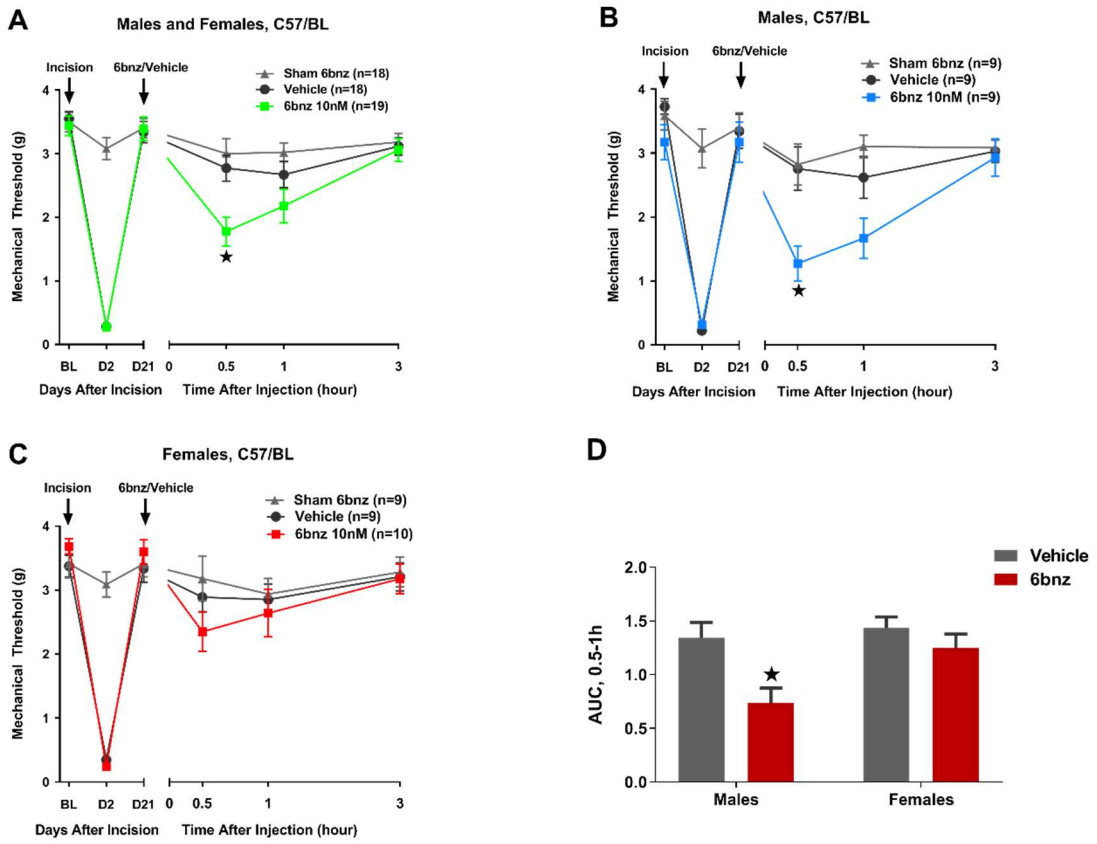

Figure 4.5. PKA activation reinstates behavioral signs of postoperative hyperalgesia in male but not female mice.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male and female C57Bl/6 mice. Intrathecal administration of (**A**) PKA activator, 6-bnz (10nmol), reinstated mechanical hyperalgesia in 21day post-operative but not sham mice. Reinstatement was elicited in (**B**) male, but remarkably not (**C**) female mice. n = 9-18. (**D**) The area under the curve (AUC, 0.5-1h) further illustrates the effect of sex on 6-bnz (10nmol) driven mechanical hypersensitivity in the ipsilateral hindpaw**.** ★$p < 0.05$ 6-bnz vs. vehicle or sham. Data represented as mean +/- SEM. Bonferroni's post-hoc tests following ordinary or repeated measures of 2-way ANOVA.

Next, we tested the hypothesis that PKA is necessary for the LS that is masked by KOR. To answer this question, we pre-administered H-89 (10nmol, i.t.), a selective inhibitor of PKA, 15 min prior to LY2456302 (10µg) delivery. Figure **4.6** illustrates that PKA inhibition by H89 prevented LY2456302-induced hyperalgesia (drug vs. time interaction, $F(18,264) = 6.73$, $p<0.001$, Table 4.2, Figure 4.6A) in 21PIM mice. Corroborating with our previous data, H-89, but not the vehicle, prevented LY2456302-induced reinstatement of hyperalgesia exclusively in male mice (drug vs. time interaction; $F(18,120) = 2.76$, $p=0.001$, Table 4.2, Figure 4.6B), which is further illustrated by the AUC (1-3h) analysis (drug vs. time interaction, $F(18, 120) = 4.99$, $p<0.0001$ for males and $p=0.69$ for females Bonferroni multiple comparisons, Table 4.3, Figure 4.6D). Together these results suggest that PKA contributes to postoperative LS in males, but not females.

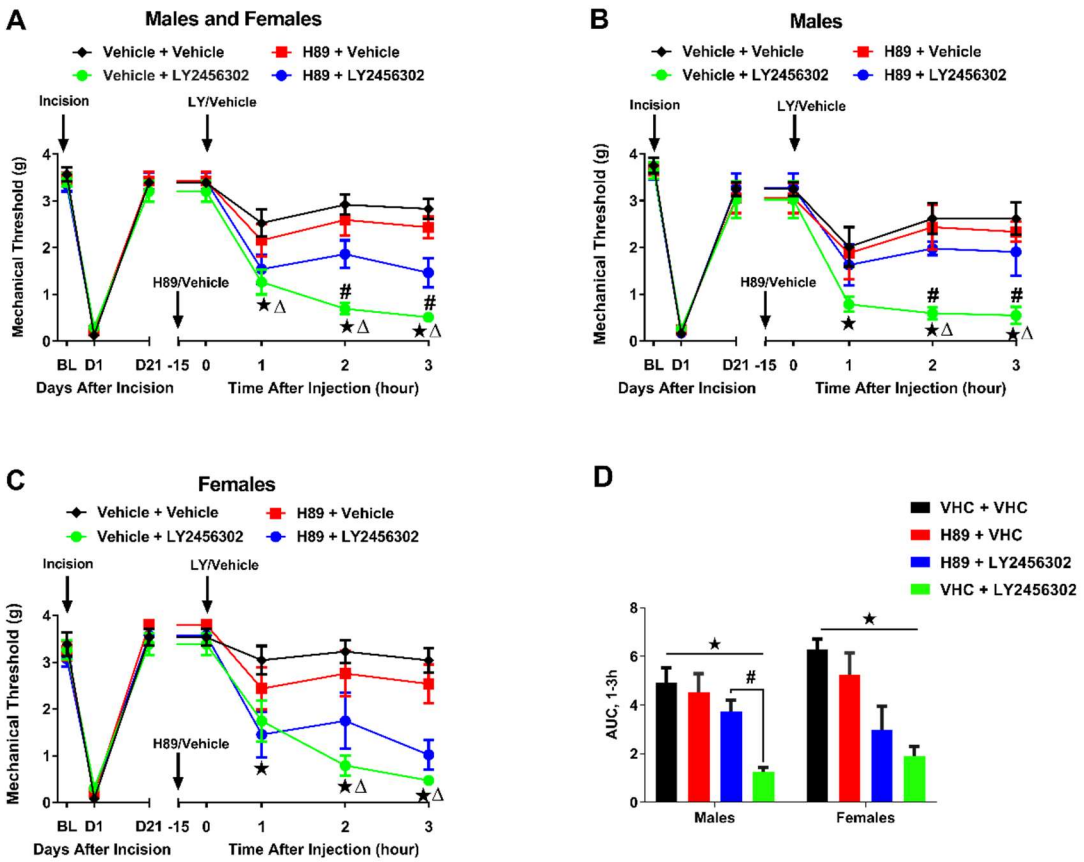

Figure 4.6. PKA inhibition prevents the reinstatement of KOR antagonist-induced postoperative hyperalgesia in male mice.

(A) Pre-administration of H89 (10nmol) prevented LY2456302 (LY, 10µg)-induced reinstatement of mechanical hypersensitivity in 21-day postoperative mice. However, when the results were stratified by sex, PKA inhibition reversed LY2456302 -induce reinstatement (**B**) only in male, (**C**) but not female mice. (**D**) The area under the curve (AUC) further illustrates that LY2456302 induced reinstatement in both sexes, but H89 prevented LY2456302 reinstatement only in male mice. n= 6-12. ★ $p < 0.05$ vehicle + LY2456302 vs. vehicle + vehicle. # $p<0.05$ H89 + LY2456302 vs. vehicle + LY2456302. Δ $p<0.05$ VHC + LY2456302 vs. H89 + VHC. Data represented as mean +/- SEM. Bonferroni's post-hoc tests followed repeated measures 2-way ANOVA.

4.4.4 Epacs activation reinstates behavioral signs of postoperative hyperalgesia in male and female mice

Cumulative evidence indicates that Epacs are required for the development of persistent hyperalgesia after inflammation or tissue injury (Wang et al., 2013; Gu et al., 2016; Singhmar et al., 2016; Matsuda et al., 2017; Singhmar et al., 2018) and LS (Fu et al., 2019) . Thus, we proposed that Epacs signaling drives LS that is masked by spinal KOR activity. To test whether Epacs mediates the pronociceptive effects of LS, we i.t. administered the Epac selective activator 8-CPT at a dose (3nmol) previously determined in our laboratory (Fu et al., 2019). **Figure 4.7A** illustrates that 8-CPT precipitated mechanical hyperalgesia in both male and female 21 d PIM mice (drug vs time interaction, $F (6, 99) =5.27$, $p<0.0001$, Table 4.2). Three-way ANOVA revealed significant effects of 8-CPT ($p=0.01$, Table 4.4) in 21d PIM mice. Subsequent two-way ANOVA analyses shown 8-CPT effects in both sexes (drug vs time interaction, $F (6, 54) =3.01$, $p=0.01$, $F (6, 45) =3.60$, $p=0.005$, for males and females respectively), but not sham controls (**Figures 4.7.B-C**). As illustrated in **Figure 4.7.D** AUC (0.5-1h) analysis revealed the significant effects of 8-CPT ($F (6, 45) = 3.60$, $p=0.005$, Table 4.3) on the reinstatement of mechanical hypersensitivity in male and female mice. These data demonstrated that Epac signaling promotes persistent postoperative latent hyperalgesia in rodents that is under inhibitory control of KOR tonic signaling.

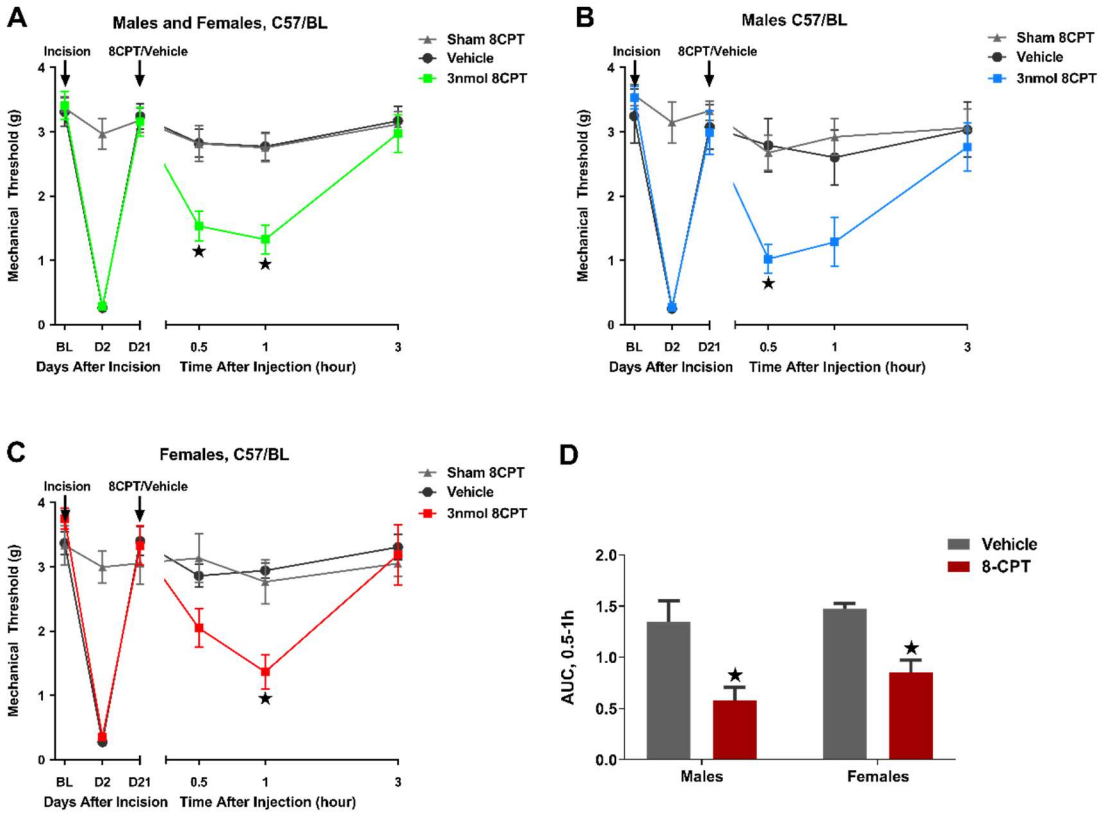

Figure 4.7. Epacs drives postoperative latent sensitization in male and female mice

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male and female C57Bl/6 mice. We used the EPAC activator, 8-CPT, to determine whether surgical incision sensitizes EPAC signaling. (**A**) Intrathecal administration of 8-CPT (3nmol) reinstated mechanical hypersensitivity three weeks after surgical incision in (**B**) male and (**C**) female mice. (**D**) The area under the curve (AUC, 0.5-1h) further illustrates the effects of sex on (8-CPT 3nmol) on mechanical hypersensitivity in the ipsilateral hindpaw**.** n = 6-12 per group. Data represent mean +/- SEM. ★ $p < 0.05$ 8-CPT vs. vehicle or sham. Data represented as mean +/- SEM. Bonferroni's post-hoc tests following ordinary or repeated measures of 2-way ANOVA.

4.4.5 Prolonged kappa opioid receptor signaling silences behavioral signs of postoperative LS via Epacs in male and female mice

Lastly, we investigated whether KOR-masked LS is driven by the AC1 downstream proteins $Epac_{1-2}$. We performed behavior pharmacological experiments using a non-selective Epac inhibitor ESI-09 (10µg) (Almahariq et al., 2013) delivered intrathecally 15min prior to LY456302 (10µg) i.t. administration in 21d PIM mice. We found that ESI-09 administration prevented the mechanical hyperalgesia induced by LY2456302 (drug vs. time interaction, $F(18, 264) = 5.13$, $p<0.001$, **Figure 4.8A**) in both male (drug vs. time interaction, $F(18, 120) = 3.99$, $p=0.0005$, **Figure 4.8B**) and female mice (drug vs. time interaction, $F(18, 120) = 2.21$, $p=0.05$, **Figure 4.8C**). The effects of ESI-09 on LY2456302-induced reinstatement of hyperalgesia are further illustrated at the AUC (1-2h) analysis (Table 4.3, **Figure 4.8D**). Together, our findings indicate that $Epac_{1-2}$ is a mutual contributor to latent postoperative hyperalgesia in male and female mice. Moreover, AC1-cAMP-Epac–dependent LS is silenced by endogenous KOR long-lasting signaling.

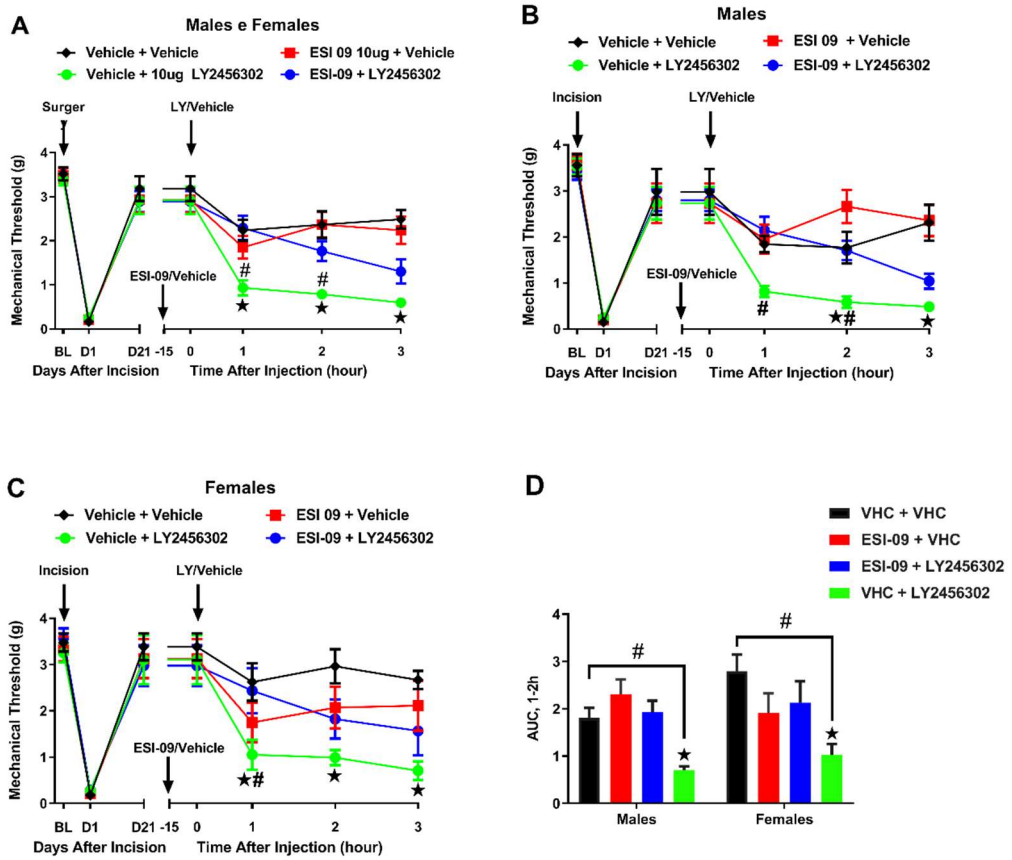

Figure 4.8. Pharmacological inhibition of Epac1-2 prevents the reinstatement of KOR antagonist-induced postoperative hyperalgesia.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male and female C57Bl/6 mice. We pre-administered the non-selective EPAC inhibitor ESI-09 before LY2456302. Intrathecal administration of ESI-09 (10µg) prevented LY2456302-induced mechanical hyperalgesia in 21day post-operative mice (**A**). This was true in both (**B**) male mice, and (**C**) female mice. (**D**) The area under the curve (AUC, 1-2h) illustrates the effects of ESI-09 (10µm) on LY2456302-induced reinstatement of mechanical hypersensitivity on both sexes**.** n = 6-12 per group. ★ $p < 0.05$ LY2456302 compared to vehicle. # $p < 0.05$ LY2456302 + vehicle compared to ESI-09 + LY2456302 represented as mean +/- SEM. Bonferroni's post-hoc tests following measures of 2-way ANOVA.

4.5 Discussion

4.5.1 Latent sensitization is driven by AC1 signaling in the dorsal horn

As a $G_{i/o}$ receptor, KOR activation promotes analgesia via adenylyl cyclase signaling inhibition (Chen et al., 1993; Bruchas and Chavkin, 2010). Similar to MOR signaling, withdrawal from chronic administration of KOR agonists leads to AC1 superactivation (Avidor-Reiss et al., 1995; Avidor-Reiss et al., 1997). AC1 is a key modulator of pain-related neuronal plasticity (Zhuo, 2012), as it links NMDAR activation to pronociceptive cAMP signaling effectors increasing susceptibility to chronic pain (Taylor and Corder, 2014). In addition, selective blockade of NMDAR precludes Nor-BNI -precipitated hyperalgesia after recovery of postsurgical pain (Campillo et al., 2011), indicating a role of NMDAR in postoperative LS. Thus, we hypothesized that KOR tonic activity silences postoperative hyperalgesia via inhibition of AC1 pronociceptive signaling, impeding the development of persistent postsurgical pain.

We found that genetic deletion of AC1 attenuates the degree of postoperative hyperalgesia, accelerates the recovery of baseline thresholds in males and reverses LY2456302-induced reinstatement of mechanical hypersensitivity on postoperative day 21 in both sexes. Similarly, pharmacological inhibition of AC1 prevents KOR-antagonist mediated hyperalgesia. Moreover, spinal nociceptive transmission induced by KOR blockade was markedly reduced in AC1KO mice compared to WT littermates. Collectively, our results indicate that AC1 is necessary for the development of latent postsurgical hyperalgesia. These results are in agreement with previous studies that suggest that AC1 is required for the establishment of pain-related sensitization (Xu et al., 2008a; Yamanaka et al., 2017) and modulates inflammatory and persistent pain states, (Wei et al.,

2002; Wang et al., 2011; Corder et al., 2013), including the latent inflammatory pain that is masked by MORCA (Corder et al., 2013).

It is well accepted that AC1 is required for the induction of spinal LTP (Wang and Zhuo, 2002; Wang et al., 2011). KOR pharmacological blockade notably reduced but did not erase spinal nociceptive transmission assessed by pERK expression in the dorsal horn of AC1 mutant mice. Other AC isoforms besides AC1 may also contribute to LS. It is conceivable that AC1 gene deletion leads to a compensatory upregulation of different AC isoforms such as its calcium-calmodulin counterpart AC-8 (Wei et al., 2006). This is in agreement with previous studies showing that AC-8 contributes to a lesser extent to LTP (Xia and Storm, 1997) and the development of inflammatory pain (Wei et al., 2002). Despite a possible effect of AC8, our findings collectively support the notion that AC1 is the critical regulator of LS. Together, our data strongly suggest that AC1 is required for persistent nociceptive sensitization within the spinal nociceptive circuits. Therefore, we propose that prolonged spinal KOR activity negatively regulates AC1 pronociceptive pathways after surgery, which in part, keeps LS within a state of remission.

4.5.2 Sex differences in the latent sensitization is driven by a PKA-dependent pathway: PKA-mediated LS in females but not males

The canonical cAMP-PKA pathway is critically involved in the development (Taiwo et al., 1989; Malmberg et al., 1997; Yajima et al., 2003) and maintenance of hyperalgesia after inflammatory injury (Sluka, 1997; Aley and Levine, 1999; Aley et al., 2000; Tumati et al., 2011), or prolonged opioid exposure (Tumati et al., 2011; Araldi et al., 2015; Chen et al., 2018b). Recent data from our laboratory demonstrates that prolonged PKA activation also participates in the pain-related sensitization (LS) associated with

persistent inflammation and nerve injury (Fu et al., 2019). However, these studies focused exclusively on males. Therefore, we assessed whether PKA persistent activity also contributes to postoperative latent sensitization masked by KOR signaling on both sexes.

Strikingly, we found that PKA activation failed to reinstate mechanical hypersensitivity in female mice three weeks after paw incision. In addition, we demonstrated that PKA inhibition using H-89 reversed the pronociceptive effects of LY2456302 only in injured male, but not female mice. These results imply that there are important sex differences in the cellular mechanisms underlying LS. To the best of our knowledge, we are the first to evaluate the direct contribution of PKA signaling in persistent postoperative pain. Our observations, however, are in agreement with findings in different chronic pain models. For example, a recent study conducted in female mice suggests that the development of hyperalgesic priming (LS) and consolidation of chronic musculoskeletal pain require the activation of the PKC-ERK pathway in the spinal cord, but is independent of PKA signaling (Chen et al., 2018c).

One possible mechanism for the lack of sensitivity to PKA in females after injury is the negative regulation of PKA by estrogen. Levine and colleagues found that in contrast to males, epinephrine-induced hyperalgesia in the spinal cord is independent of protein kinases activity in normally cycling or gonadectomized females receiving hormonal replacement. However, PKA signaling was restored in ovariectomized animals (Dina et al., 2001). Conversely, an in vitro study revealed that membrane estrogen receptors regulates ERK phosphorylation, but do not influence PKA activity (Nag and Mokha, 2014).

Our findings indicate that postoperative spinal sensitization is independent of PKA signaling in female mice, although, these results should be interpreted with caution. First,

the doses used in our studies were based on dose-response curves established in pharmacological studies in male mice. Therefore, the pharmacokinetics of these drugs might differ between male and female mice. Second, although we failed to demonstrate the contribution of PKA in the remission phase of LS, we cannot exclude the participation of cAMP-PKA signaling in the induction of acute postoperative pain. Recent evidence indicates that PKA is required for the induction of hippocampal LTP in females, but not male mice (Jain et al., 2019). Similarly, PKA activity in nociceptors is required in acute and persistent inflammatory models in females (Aley and Levine, 1999; Araldi et al., 2015). Thus, it is conceivable that as inflammation remits and spinal nociceptive plasticity persists, there is a shift from a PKA dependent to a PKA-independent mechanism associated with the activation of distinct signaling pathways. For example, a studies in female mice showed that inflammation alters G-protein-coupled receptor kinase 2 (GRK2) protein levels in the spinal cord and leads to cross talk of GRK2 with Epac and ERK signaling, independently of PKA leading to persistent hyperalgesia (Eijkelkamp et al., 2010; Huang and Gu, 2017). Alternatively, the activation of parallel cAMP pathways may be sufficient to produce persistent hyperalgesia after surgery in females.

4.5.3 Contribution of Epacs to latent postoperative pain

We found that Epac activation by 8-CPT-cAMP (Rehmann et al., 2003) restores mechanical hypersensitivity at least 21 days after surgery in both male and female mice. We speculate that similar to AC1 (Corder et al., 2013), tissue injury leads to superactivation of Epacs. This would fit with previous observations that Epac1 and Epac2 expression are markedly increased 14 days after surgical incision in the DRG and dorsal horn in a subset

of small non-peptidergic fibers (Cao et al., 2016; Matsuda et al., 2017), although expression does not equal Epac activity, and Epac activity was not measured in these studies.

We found that the Epac1-2 inhibitor ESI-09 prevented the ability of LY2456302 to reinstate hyperalgesia, indicating that Epac is required for the maintenance of LS. Our findings are in agreement with a previous study demonstrating that ESI-09 prevents hyperalgesia induced by the administration of inflammatory mediators 14 days after surgical incision, but did not affect acute postoperative pain (Matsuda et al., 2017). The effects of Epac in persistent postsurgical hyperalgesia in these studies were mediated by p38MAPK (Matsuda et al., 2017) and lead to the translocation of PKC in primary afferents (Hucho et al., 2005), suggesting the contribution of PKC in LS. Moreover, the activation of specific PKC isoforms by Epac signaling was recently demonstrated *in vitro* (Goode and Molliver, 2019). However, the role of downstream effectors of Epac in LS remains to be determined.

Collectively our data indicate significant sex differences in latent postoperative pain. We propose that an AC1-cAMP-PKA mechanism drives LS in males, while AC1-cAMP–Epac signaling is a common mechanism underlying LS in both males and females. Both pathways are kept under remission by sustained KOR activation.

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CHAPTER 5 – Final Discussion, Conclusions, and Future Directions

5.1 General Discussion

In this dissertation we aimed to investigate the contribution of opioid receptor subtypes to postoperative latent sensitization and cellular mechanisms that drive LS in male and female mice. Our findings indicate that spinal MOR and KOR, but not DOR mask LS. Furthermore, our data indicate that surgical incision activates AC1, PKA, and Epac pain facilitatory processes, which in turn, is opposed by a long-lasting spinal KOR inhibitory signaling. One of our main findings is that spinal KOR activity differentially regulates LS in male and female mice. Moreover, cAMP dependent pathways drive postoperative LS in a sex-dependent manner. These findings have important implications for the development of new therapeutics targeting postsurgical pain.

5.1.1 Kappa opioid system: paradoxical effects in pain modulation

The contribution of the KOR-dynorphin system in pain modulation has been extensively investigated in rodents and human subjects in the past decades. According to the site of action in the pain neuroaxis, KOR agonism result in analgesia or hyperalgesia. For instance, peripheral restricted (Snyder et al., 2018), and KOR agonists injected into the RVM (Hurley and Hammond, 2000; Schepers et al., 2008a) promote analgesia after injury; while KOR activation in the central amygdala leads to hyperalgesia in primed animals (Xie et al., 2017).

At the spinal cord level, dynorphin peptide administration leads to analgesia in several models of acute pain, an effect that is mediated by coupling to KOR (Spampinato and Candeletti, 1985; Podvin et al., 2016). In contrast, in chronic inflammatory and nerve

injury models dynorphin elicits hyperalgesia/allodynia via non-opioid pathways, possibly through direct or indirect interaction with NMDA or bradykinin receptors (Vanderah et al., 1996; Tan-No et al., 2002; Lai et al., 2006; Luo et al., 2008). Our results demonstrated that blockade of spinal KOR with long- or shorter-acting selective compounds during LS reinstates hyperalgesia over one year after surgical incision, which is accompanied by increased nociceptive spinal transmission. Thus, we provided compelling evidence that spinal KOR signaling elicits a long-lasting postoperative endogenous antinociceptive effect. Therefore, it is reasonable to speculate that spinal KOR masks nociceptive sensitization in the dorsal horn after surgery through increased constitutive activity or dynorphin-independent signaling. This hypothesis was not tested in this dissertation; however, it is in agreement with the study of Walwyn and colleagues that showed that long-acting KOR antagonists produce hyperalgesic effects at similar magnitude on 21-day CFA WT and pre-prodynorphin deficient mice (Walwyn et al., 2016). In this line of thought, systemic administration of the KOR antagonist Nor-BNI reinstates mechanical hyperalgesia in surgical incised and opioid-treated mice with no evidence of changes in spinal dynorphin levels (Campillo et al., 2010). Moreover, constitutive activity of KOR was recently demonstrated in vivo in a preclinical model of stress-induced drug relapse. Collectively, our results suggest that not only MOR_{CA} but also by long-lasting KOR signaling may preclude the transition from acute to chronic postoperative pain.

5.1.1.1 Kappa opioid receptors as a potential therapeutic target for chronic pain and addiction

The “holy grail” of opioid pharmacology is the development of opioids devoid of the potentially fatal, and undesirable addictive effects of MOR. KOR is an attractive target

since its activation does not elicit MOR-related adverse effects such as respiratory depression, nausea, constipation, and has lower abuse potential (Chavkin, 2011). While euphoria results exclusively from MOR activation in the mesocorticolimbic system, KOR activation in the brain generates dysphoria and psychotomimetic effects (White and Roth, 2012). Thus, despite the robust postoperative analgesic effects of full KOR agonists (Pande et al., 1996a), the psychotomimetic effects in therapeutic dosages had refrained these drugs from further use in the clinical setting (Pfeiffer et al., 1986).

Therefore, in the field of KOR therapeutics, efforts have been concentrated in the identification of new compounds that work through selective signaling pathways (bias agonism) independent of β-arrestin and MAPK pathways entailed to the aversive effects of kappa (Land et al., 2008; Chavkin, 2011). To date, these compounds were tested predominantly in vitro (White et al., 2015; Schattauer et al., 2017). Future studies in vivo may shed some light on the analgesic efficacy of those compounds. Emerging research have focused on the peripheral analgesic properties of KOR as potential treatment for visceral pain (Stein et al., 2009; Snyder et al., 2018), however the clinical applicability of these drugs has been contested as at higher doses peripheral restricted agonists can still cross the blood brain barrier and elicit similar CNS adverse effects (Nielsen et al., 2015; Naser and Kuner, 2018).

Alternatively, KOR antagonists have been proposed as potential treatment for nociplastic disorders triggered by stress, such as migraines (Xie et al., 2017). As previously discussed, Nor-BNI and other long-lasting KOR antagonists due to its extended duration of action and likely cumulative effects are unsuitable for clinical use. The short-acting drugs used at the abovementioned studies are at early stages of development and

have been tested so far only in preclinical studies. The results of these studies, however, are very preliminary, due to the paucity of translational models of chronic stress. Moreover, stress is only one of the initiator factors and comorbidities of these chronic pain conditions and are not easily identified clinically. One of the KOR antagonists used in this dissertation LY2456302 (currently known as CERC501) is currently in phase II clinical trials for major depression. Despite the uncertainty of short KOR antagonists to manage chronic pain, they may represent a good adjuvant or monotherapy for the treatment of post-traumatic stress and substance use disorders (Rogala et al., 2012; Reed et al., 2018).

5.1.2 Postoperative latent sensitization may be silenced by synergism of the opioidergic and other GPCR systems

In this dissertation, we demonstrated that LS is maintained quiescent by the long-lasting activity of MOR and KOR after surgery. Our laboratory previously demonstrated that MOR increased constitutive activity (MOR_{CA}) after injury that persistently represses hyperalgesia (Corder et al., 2013). However, LS is also opposed by non-opioidergic receptor systems. For example, blockade of NPY-Y1R reinstates mechanical and thermal hyperalgesia in 21-day mice that received intraplantar CFA and 28 days after nerve injury (Solway et al., 2011; Fu et al., 2019). Remarkably, pharmacological blockade of Y1R, MOR and KOR reinstates hyperalgesia in a similar magnitude. These observations raised the question of whether NPY and opioid systems act in concert through a synergistic or additive effect. Ongoing studies in our laboratory indicate endogenous synergism between these two receptor systems (dos Santos et al., unpublished findings), however, whether they are co-expressed in the same population of dorsal neurons remains to be determined. Functional interaction between GPCR has implications in pharmacology. For example,

the formation of homo and heterodimerization between GPCR has been described primarily *in vitro*, but still controversial (Al-Hasani and Bruchas, 2011). A recent study evaluated the co-expression of MOR and DOR in vivo (Wang et al., 2018). An intriguing hypothesis is that injury increases the formation of pain modulatory receptors systems heterodimers to potentiate inhibitory signals and to combat pronociceptive effects during LS.

5.1.2.1 Tonic cannabinoid receptor-1 and cannabinoid receptor -2 activity suppresses postoperative hyperalgesia

Endocannabinoid receptor systems are implicating in the modulation of postsurgical pain (Alkaitis et al., 2010). Similar to MOR and KOR, we found that CB1 and CB2 receptors blockade reinstates mechanical hypersensitivity in a dose-dependent manner three weeks after surgical incision (data not shown), but in a lesser magnitude. We then investigate whether CB1 receptors exert their analgesic effects after surgery via constitutive activity. We found that the neutral antagonist AM4113 failed to reverse the hyperalgesic effects of the inverse agonist rimonabant in the dorsal horn. Our results suggest that CB1 is activated by their endogenous ligand after surgery to modulate postsurgical hyperalgesia. Functional interactions between CB1 and KOR has been described. Moreover, activation of CB1 and MOR heterodimers markedly reduced ERK signaling compared to the individual activation of these receptors (Al-Hasani and Bruchas, 2011). Thus, it is possible that MOR and/or KOR and CB1 act in synergism to modulate LS after surgical incision.

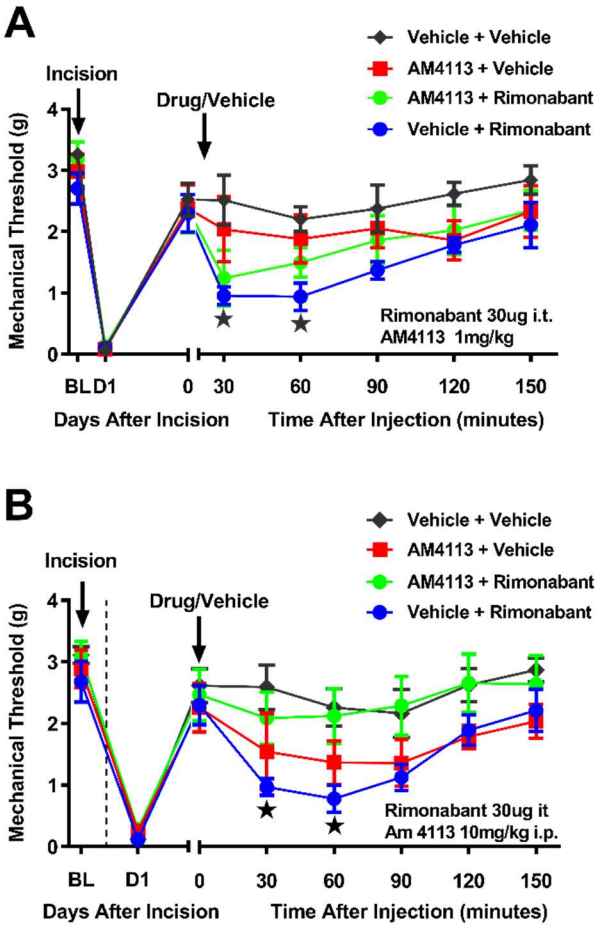


Figure 5.1. Spinal administration of CB1 inverse agonist reinstates mechanical hypersensitivity in surgical incised mice.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male C57Bl/6 mice. The inverse agonist Rimonabant (30µg, i.t.) induced reinstatement of hyperalgesia (n= 6-7 per group) in male mice effect that was not abolished by the pre-administration of the neutral antagonist AM 4113 in either 1mg/kg (i.p.) **(B)** or 10mg/kg (i.p.) dose**.** ★ $p < 0.05$ vehicle + Rimonabant compared to vehicle + vehicle. Bonferroni's post-hoc tests following repeated measures of 2-way ANOVA. Data represented as mean +/- SEM.

5.1.3 Sex differences in Latent Sensitization

In this dissertation, we explored the effects of spinal KOR signaling following surgical incision in a preclinical model of postoperative pain. Our results demonstrated that KOR prolonged signaling within the spinal nociceptive system promotes postoperative endogenous analgesia in rodents. As previously emphasized, KOR agonists were previously tested for postoperative pain in the clinical setting with limited analgesic efficacy due to its narrow therapeutic window (Pfeiffer et al., 1986). Interestingly enough, selective KOR agonists and mixed-action partial agonists were more efficacious postoperatively in women and female rodents (Gear et al., 1996a; Gear et al., 1996b; Pande et al., 1996a). These findings substantiate our observations that endogenous KOR signaling are more pronounced in female mice after surgery. Sex differences in opioid analgesia is attributed to gonadal hormones regulation of spinal KOR expression and signaling, and genetic predisposition.

A remarkable finding in our studies is that c-AMP dependent signaling after surgery differs between sexes. In females, LS is driven by a PKA independent pathway, while AC1-Epac is a common mechanism in both males and females. One possibility is that gonadal hormones negatively regulate PKA in females. Alternatively, it is conceivable that cAMP-PKA signaling is present in the first stages of the development of postoperative hyperalgesia, and as hyperalgesia progresses a shift from PKA to other pathways (i.e. Epac) occurs due to the influence of other kinases. Nevertheless, the investigation of differences in pain signaling pathways is important to determine possible mechanisms associated with the higher predisposition of females to develop chronic pain, and sex-specific therapeutic targets.

5.1.4 AC1 and Epac are potential therapeutic strategies for the management of chronic postoperative pain

Our data and previous studies in our laboratory indicate that AC1 and Epac are required in the transition from acute to persistent pain states in rodents. AC1 and Epac superactivation are common mechanisms in LS opposed by opioid and other receptor systems (Corder et al., 2013) (Fu W et al., in revision), and are observed in both male and female mice. The window of activation of these proteins after surgery, however; remains unclear. An interesting feature of postoperative pain is the presence of a clear etiology that allows the distinction between acute pain and the progression to persistent pain states, which is a challenge of most chronic pain conditions. AC1 is an interesting candidate as its inhibition not only promotes analgesia but also combats opioid dependence. Moreover, AC1 is highly expressed in the brain and contributes to the regulation of synaptic plasticity, learning, and memory. Thus, a certain degree of cognitive impairment is an expected adverse effect in future clinical trials. The development of drugs targeting AC1 downstream targets, i.e., Epac protein may circumvent these limitations.

Moreover, current studies in our laboratory conducted by Dr. Liping Zhang also demonstrate that systemic administration of the Epac inhibitor, ESI-09, reduces mechanical hypersensitivity and ongoing pain in a preclinical model of chronic neuropathic pain. These observations reinforce the analgesic potential of Epacs and their indication for the management of chronic pain. In summary, our studies indicate that pharmacological AC1 and Epac inhibitors silence postoperative LS. Therefore, drugs targeting AC1 and Epacs may represent good alternatives to manage chronic postoperative

pain, avoiding the prolonged use of opioids and their deleterious side effects such as tolerance, dependence, and addiction.

5.2 Conclusions

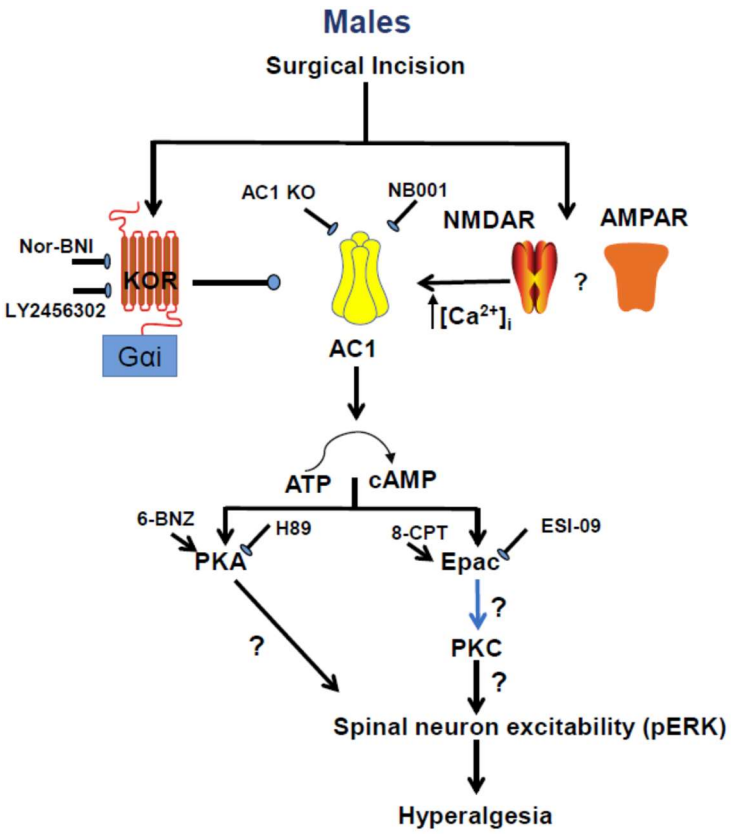
In this dissertation, we employed a preclinical model of chronic postoperative pain that is ideally suited to assess candidate-signaling molecules involved in the maintenance of pain-related plasticity and the receptor systems involved in postoperative latent pain sensitization (LS). We demonstrated that pharmacological blockade of KOR (or MOR) markedly restores mechanical hypersensitivity and spinal sensitization at least three weeks after surgical intervention. Furthermore, KOR antagonism reveals a silent, persistent sensitization mediated by AC1 and downstream cAMP-dependent signaling pathways. **Figure 5.2** summarizes this work.

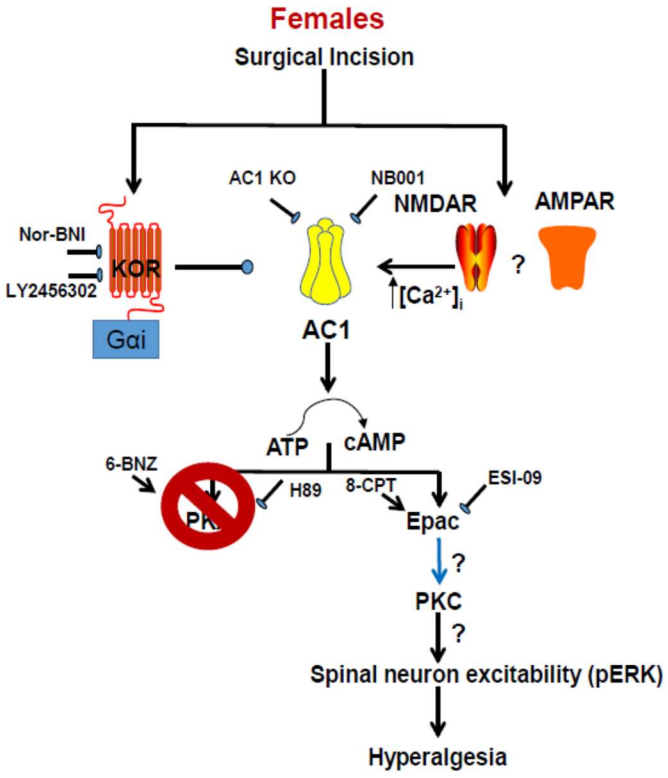
Our work also indicates that surgical incision promotes long-lasting MOR and KOR endogenous analgesia in the dorsal horn of the spinal cord that persists for at least one year after injury. We reasoned that long-term KOR activation represses a prolonged AC1 – cAMP – PKA and/or AC1- cAMP - Epac spinal pronociceptive signaling which, in part, maintains LS within a state of remission. Thus, our observations suggest that any disruption of spinal MOR_{CA} or KOR signaling, i.e., caused by significant stressful events or additional injury, may potentiate LS pronociceptive signals leading to persistent postoperative pain. Therefore, we propose that spinal KOR activation precludes the transition from acute to chronic postoperative pain via inhibition of AC1 and cAMP-dependent pathways.

We further extended the literature assessing the influence of biological sex on LS. We provided evidence of a small magnitude, but significant sex difference in endogenous

KOR-mediated postoperative analgesia. Our data suggests that females possess enhanced spinal KOR signaling after tissue injury, which may explain, at least in part, the observed sexual dimorphism in the responsiveness to KOR agonists administered postoperatively in clinical trials (Gear et al., 1996a; Gear et al., 1996b; Pande et al., 1996b; Pande et al., 1996a). Intriguingly, this work further demonstrates a sexual dimorphism in the c-AMP dependent pathways involved in the induction and maintenance of pain-related plasticity following surgical intervention, wherein male both AC1-cAMP-PKA and AC1- cAMP-Epac drive LS, while in females LS is driven by AC1-cAMP-Epac, i.e., a PKA-independent mechanism. Moreover, selective AC1 and Epac inhibitors reverse the reinstatement of mechanical hypersensitivity induced by KOR antagonist in both male and female mice while PKA selective inhibition precludes LY2456302-reinstatement of mechanical allodynia only in male mice, confirming that both AC1-cAMP pathways are under the inhibitory control of KOR signaling. Our observations raise the prospect that differences in signaling pathways modulating spinal nociceptive plasticity may help to explain the higher susceptibility of females to develop chronic postoperative pain.

We present Epac as a new potential therapeutic target to manage chronic postoperative pain, and AC1 inhibitors as a possible effective treatment for postoperative pain. As noted, AC1 inhibition accelerates the recovery from postoperative pain in males, and the effects of AC1 inhibition are more noticeable as hyperalgesia persists. Therefore, the identification of sex-specific therapeutic targets should be taken into account as new therapeutics progress into clinical trials.


Males
Surgical Incision
AC1 KO
NB001
NMDAR
AMPAR
Nor-BNI
KOR
LY2456302
Gαi
?
[Ca2+]i
AC1
ATP
cAMP
6-BNZ
H89
8-CPT
ESI-09
PKA
Epac
?
PKC
?
?
Spinal neuron excitability (pERK)
Hyperalgesia


Females
Surgical Incision
AC1 KO
NB001
NMDAR
AMPAR
Nor-BNI
KOR
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cAMP
6-BNZ
PKA
H89
8-CPT
ESI-09
Epac
?
PKC
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Spinal neuron excitability (pERK)
Hyperalgesia

Figure 5.2. A conceptual model of pronociceptive signaling pathways suppressed by endogenous KOR long-lasting activity in the dorsal horn following surgery in male and female mice.

Arrows indicate activation; Line Bottom indicates inhibition; Blue Arrow indicates future directions. For abbreviation, please see appendix 1.

5.3 Future Directions

1. In chapter 3**,** our behavioral pharmacological experiments revealed that KOR signaling maintains LS within a state of remission after surgical incision. Both Nor-BNI and LY2456302 dose-dependently (in a ηM - μM range) reinstated mechanical hypersensitivity at postoperative day 21. *In vitro* and *in vivo* studies show that Nor-BNI has a slow onset (hours –systemically (Munro et al., 2012); 30 minutes i.c.v. (Horan et al., 1992), and transient MOR signaling effects during the first hours of action. A recent animal and clinical studies also indicated that higher doses of LY2456302 might have off-target effects at MOR (Reed et al., 2018). To date, the literature had evaluated the systemic effects of LY. For the best of our knowledge, we are the first to assess the i.t. effects of this compound. Thus, despite the low dosages used in our studies, future experiments using KOR KO mice would help to further ratify our findings. Alternatively, selective deletion of spinal KOR (i.e.) using dynorphin-saporin i.t. administration before plantar incision could also be used. Moreover, behavioral pharmacological trials testing whether LY2456302 or Nor BNI at 1 - 10μg dose reverts the effects of equi-antinociceptive i.t. doses of MOR agonists, i.e. morphine or DAMGO, after surgical incision can also help to confirm the selectively of KOR signaling in our studies.

2. We found that spinal MOR and KOR blockade reinstates hyperalgesia, however; we cannot exclude pre-synaptic effects of these receptors. Indeed, recent data based on conditional MOR KO (MOR flox/Nav 1.8 mice) mice indicates that MORs located on central terminals of primary afferent neurons exert inhibitory effects during the remission phase of LS (Severino et al., 2018). Similarly, a recent elegant study from the Ross group characterized KOR expression and function on primary afferents. They showed that KOR

expressed at peripheral and central terminals of peptidergic neurons are involved in the control of mechanical postoperative pain (Snyder et al., 2018). Therefore, future studies using in situ hybridization, KOR conditional KO (Cai et al., 2016) or chemogenetic approaches may clarify the precise population of spinal KOR-expressing neurons responsible for the endogenous control of persistent postoperative pain.

3. We tested only one or two dosages of DOR selective antagonist on figure 3.2 since higher doses of these drugs (i.e $TIPP_{(\Psi)}$ 20ug, and Naltrindole10ug) elicited side effects such as scratching, biting of back and paws, spontaneous flinching of the paws, tachypnea, excessive grooming and jumping that prevented further testing. Moreover, we tested the effect of $Tipp_{(\Psi)}$ 10ug i.t. on a separate cohort of 21-day postoperative female mice (data not shown) and observed similar effects to the male mice (lack of reinstatement of hyperalgesia). Taking into account possible pharmacokinetic differences of the drugs in male and female animals, future studies using higher doses of selective DOR antagonists in females and DOR KO mice may be beneficial to support the lack of effect of DOR activity in LS found in our studies and rule out a possible sex dimorphism in DOR-mediated LS.

4. We evaluated sex differences exclusively in stimulus-evoked (mechanical) pain. Spinal KOR neurons play a major role in the modulation of thermal pain (Martin et al., 2003; Snyder et al., 2018), therefore; dose-response studies and studies evaluating sex differences in thermal hypersensitivity (i.e., Hargreaves' method (Hargreaves et al., 1988) are warranted. In addition, the sex differences reported in postoperative pain in clinical studies were based on patients' self-report. Thus, potential sex differences can also be assessed using preclinical assessment of ongoing pain (i.e., CPP or CPA) or facial expression as a

surrogate for the visual analog scale used in the clinical trials. Furthermore, investigations of the effects of KOR antagonists in supraspinal sites involved in pain modulation such as the RVM (Ossipov et al., 2014) on 21 day postoperative mice should clarify whether the discrete sex differences observed in our studies are due to spinal or supraspinal KOR modulation of LS.

5. As gonadal hormones positively regulate the expression and function of KOR (Harris et al., 2004; Chartoff and Mavrikaki, 2015), the outcomes observed in our studies may be influenced by the phase of the estrous cycle in female subjects. Therefore, a major limitation in our sex difference studies is that we did not assess the effect of gonadal hormones in postoperative nociceptive behaviors. Future studies should evaluate the behavioral effects of KOR antagonists in different stages of the estrous cycle using vaginal smear in regularly cycling females after surgery. Since, pain may affect the regular estrous cycle in females, hormone replacement studies may be also be conducted in gonadectomized animals. In addition to hormonal studies, the effect of sex chromosomes on KOR endogenous analgesia can also be investigated. Sex chromosome genes (XX vs XY) in the nociceptive pathways seem to induce changes in nociception and may be independent of gonadal hormones (Gioiosa et al., 2008). For example, XX mice present higher brain levels of prodynorphin mRNA in the brain compared to XY irrespective of sex (Chen et al., 2009). In addition, KOR agonists increase hot plate thresholds in neonatal XX mice (Gioiosa et al., 2008). Thus, sex differences in KOR/dynorphin system may also be attributable to sex chromosome complements, regardless of hormone levels.

6. The sex differences in KOR-inhibited LS may also be explained by differences in KOR density or dynorphin levels between males and females. We have not measured KOR

expression in the dorsal horn of the spinal cord after plantar incision. Recent data from our laboratory (experiments performed by Mei Lu) showed that the levels of Oprk1 (KOR gene) in the lumbar dorsal horn are significantly higher in naïve and three days after sciatic nerve injury (SNI) in female mice. Moreover, KOR gene expression significantly changes throughout the course of neuropathic pain development (up to 14 days after SNI) in females. Thus, it is reasonable to speculate that KOR expression can also differ between sexes after inflammation. KOR expression levels could be assessed in the ipsilateral vs. contralateral L3-L4 dorsal horn at baseline and different timepoints after plantar incision and POD-21 in male and female mice using qPCR and in situ hybridization.

7. In chapter 4, our data showed that KOR activation promotes a very long-lasting postoperative analgesia. However, the mechanisms by which KOR signaling is activated after surgery and LS stages remain unclear. Levels of dynorphin are reported to be increased after surgical incision (Campillo et al., 2010). Interestingly, dynorphin deletion does not reverse selective-KOR blockade induced-pain reinstatement after intraplantar CFA injection (Walwyn et al., 2016). Thus, one possibility is that injury leads to KOR constitutive activation. Recent reports reveal that reinstatement of drug-seeking is mediated by KOR constitutive activity in the brain following acute stress (Polter et al., 2017). However, little is known about the mechanisms that initiate constitutive signaling. *In vitro and in vivo* data indicate that agonist induced-KOR activation leads to the phosphorylation of Ser 369 residue at the KOR C-terminus tail (McLaughlin et al., 2004). Thus, constitutive activity could be assessed using validated KOR phospho-selective antibodies (Lemos et al., 2011) in WT and pro-dynorphin KO mice at different time points after surgical incision using IHC or western blots. Alternatively, pharmacological

behavioral or radioligand binding studies using i.e., Nor-BNI (KOR inverse-agonist) and 6-β-naltrexol as KOR (non-selective) neutral antagonist (Wang et al., 2007; Polter et al., 2017), can be used to test the GTP-dependent binding capacity and the ability of KOR inverse-agonist to reinstate behavioral hyperalgesia in the presence of the neutral antagonist.

8. Our behavioral pharmacological studies indicate that AC1 and downstream cAMP pathways drive LS and are inhibited by KOR signaling. Similar to MOR, chronic administration of KOR agonists leads to upregulation of AC and cAMP overproduction (Avidor-Reiss et al., 1995; Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997). Therefore, as previously demonstrated in the MOR systems (Taylor and Corder, 2014), KOR long-lasting signaling during postoperative LS may reduce AC1 super activation (cAMP overshoot). Thus, the next logical step is to measure cAMP levels after surgical incision. First, we could assess levels of cAMP in the lumbar spinal cord to test whether KOR antagonist i.t. administration leads to cAMP overshoot in 21-day postoperative mice compared to controls. As previously described, cAMP Elisa analyses can be performed in lumbar sections of both groups (Corder et al., 2013).

9. Our results demonstrated that AC1 significantly contributed to LS, which is inhibited in part, by KOR signaling. Although, it is conceivable that to a lesser extent, other AC isoforms, i.e., the Ca^{+2}-calmodulin-dependent AC, isoform – 8 (AC8) also contributes to the induction of LS after surgery, and development of hyperalgesia in females. This hypothesis is supported by a recent study *in vitro* that suggests that NB001 acts primarily through attenuation of cAMP accumulation rather than AC1 direct inhibition (Brand et al., 2013). Also, our pERK experiment showed that KOR blockade markedly reduced, but did

not eliminate spinal nociceptive transmission. Thus, behavioral pharmacological and electrophysiological studies using $AC8^{-/-}$, and/or AC1/AC8 double KO can be used to elucidate whether AC8 also participates in the induction and/or maintenance of LS. The newly developed AC1-selective inhibitor ST034307 may also be used in future studies (Brust et al., 2017).

10. We did not test in this dissertation whether KOR suppresses a NMDAR (NMDAR subtypes) – AC1 pathway. Recent data from our laboratory indicates that NMDAR are involved in the maintenance of LS unmasked by the non-selective opioid antagonist NTX. Accumulating evidence suggest that AMPA-derived calcium is the predominant post-synaptic mechanism implicated in post-incisional sensitization (Pogatzki-Zahn et al., 2017), and possibly also activates Ca^{2+} - sensitive adenylyl cyclases. Studies recently published by our laboratory indicate the contribution of AMPARs to induction and maintenance of LS 21 days after intraplantar CFA (Taylor et al., 2019). Therefore, multidisciplinary studies including the administration of AMPAR/NMDAR selective inhibitors in vivo or ex vivo, calcium imaging and biochemical approaches may help to answer whether KOR inhibits an AMPAR and/or NMDA dependent plasticity after surgery.

11. Our behavioral data indicate that persistent postoperative pain is mediated by cAMP-PKA signaling only in male mice. The lack of effect of PKA activation or inhibition in mechanical hypersensitivity in females may reflect the negative regulation of PKA gonadal hormones (Dina et al., 2001). We did not control for the female estrous cycle. Thus, it will be interesting if future studies evaluate the effect of PKA activation in 21-day postoperative

ovariectomized mice and ovariectomized animals receiving estrogen supplementation to rule out the influence of estrogen on PKA signaling in our studies.

12. Our behavioral studies suggest sexual dimorphism in cAMP-dependent pathways mediating LS. Thus, biochemical measures of EPAC (i.e., western blots or in situ hybridization) and PKA (activity assay and/or western blots) would confirm their contribution and sexual dimorphism in LS. Moreover, these studies will help to determine the expression and window of activation of these signaling molecules in the dorsal horn after injury. As PKA activates several downstream substrates such as $ERK_{1/2}$ (Kawasaki et al., 2004) and CREB (Hoeger-Bement and Sluka, 2003), it is reasonable to speculate that i.t. delivery of the PKA activator (6-bnz) in 21-day postoperative mice would show significant differences in the expression of these central sensitization markers in the dorsal horn of male compared to female mice.

13. We did not determine the specific Epac isoform involved in LS, in part, due to the lack of a selective Epac1 inhibitor. To date, only Epac 2 selective inhibitors (HJC0350) are commercially available. Alternatively, the contribution of both Epac isoforms can be evaluated using Epac1 and Epac 2 KO mice or siRNA approaches (Cao et al., 2016). Furthermore, Epac is upstream of PKC (Hucho et al., 2005; Gu et al., 2016). PKC has a central role in the induction and maintenance of spinal nociceptive plasticity (Aley et al., 2000; Dina et al., 2001). Similar to PKA, PKC also phosphorylates ERK, CREB and glutamatergic receptors. Thus, PKC is an interesting next step in the cAMP pathway to be investigated in LS. We reason that similar to Epac, PKC activation is required for LS maintenance in both sexes.

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APPENDICES

APPENDIX 1. Abbreviations

6Bnz	N6 - Benzoyladenosine- 3', 5'- cyclic monophosphate sodium salt, cAMP analog/PKA activator
8cpt	8-(4-Chlorophenylthio)-2'-O-methyladenosine 3', 5'-cyclic monophosphate sodium salt, cAMP analog/EPAC activator
AC	Adenylyl cyclase
AC1	Adenylyl cyclase type 1
AC8	Adenylyl cyclase type 8
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
AUC	Area under the curve
BL	Baseline, mechanical thresholds
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
cAMP	adenosine 3′,5′-cyclic monophosphate
CB1	Cannabinoid receptor subtype 1
CB2	Cannabinoid receptor subtype 2
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CPP	Conditioned place preference
CREB	cAMP response element binding protein
CTOP	Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH2, MOR selective antagonist

DAMGO	[D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin, MOR agonist
DPDPE	([D-Pen^{2,} D-Pen^{5}] enkephalin), DOR agonist
DAPI	4', 6-diamidino-2-phenylindole
DRG	Dorsal root ganglia
DOR	Delta opioid receptor
EC_{50}	Half-maximal effective concentration
E_{MAX}	Maximum possible effect
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular-signal-regulated kinases
GABA	Gamma-aminobutyric acid
CGRP	Calcitonin gene-related peptide
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GIRK	G-protein linked inward rectifying potassium channel
GPCR	G-protein coupled receptor
GRK2	G-protein-coupled receptor kinase 2
GTP	Guanosine-5'-triphosphate
IASP	International Association for the Study of Pain
IB4	Isolectin B4
Iba-1	Iba-1 ionized calcium-binding adapter molecule -1e
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.t.	Intrathecal

KOR	Kappa Opioid Receptor
LS	Latent sensitization/ Latent pain sensitization
LTP	Long-term potentiation
LY	LY2456302, KOR selective antagonist
MAPK	Mitogen-activated protein kinase
MOR	Mu opioid receptor
MOR_{CA}	Mu opioid receptor constitutive activity
MPE	Maximum Possible Effect
NeuN	Neuronal nuclei
NK1R	Neurokinin receptor 1
NMDA	N-methyl-D-aspartate
Nor-BNI	Norbinaltorphimine dihydrochloride, KOR selective antagonist
NTX	Naltrexone, opioid receptor non-selective antagonist
PAG	Periaqueductal gray
PB	Phosphate buffer
PBN	Parabrachial nucleus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pERK	Phosphorylated extracellular-signal-regulated kinases
PIM	Plantar Incision Model
PKA	Protein kinase A
PKC	Protein kinase C
RVM	Rostral ventral medulla

SP	Substance P
TIPPψ	H-Tyr-TicΨ[CH2-NH]Phe-Phe-OH
vF	von Frey filaments
VGCC	Voltage gated calcium channel
VGLUT	Vesicular glutamate transporter
WDR	Wide dynamic range neuron

APPENDIX 2. IASP Taxonomy – Pain nomenclature used in this dissertation

Term	Definition
Allodynia	Pain due to a stimulus that does not normally provoke pain.
Analgesia	Absence of pain in response to stimulation which would normally be painful.
Central sensitization	Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input.
Hyperalgesia	Increased pain from a stimulus that normally provokes pain.
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system.
Neuropathy	A disturbance of function or pathological change in a nerve: in one nerve, mononeuropathy; in several nerves, mononeuropathy multiplex; if diffuse and bilateral, and polyneuropathy.
Nociception	The neural process of encoding noxious stimuli.
Nociceptive neuron	A central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli.
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.
Nociceptive stimulus	An actually or potentially tissue-damaging event transduced and encoded by nociceptors.

Nociceptor	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli.
Nociplastic pain	Pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain.
Noxious stimulus	A stimulus that is damaging or threatens damage to normal tissues.
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.
Peripheral sensitization	Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields.

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Honors and Awards

2018	Fellow, American Academy of Orofacial Pain
2018	Conference Travel Award (University of Kentucky) for 17th World Congress of Pain, Boston, Massachusetts.
2018	Poster Award Neuroscience - 34th Annual BGSFN Spring Neuroscience Day 2018 CCTS Spring Conference, Lexington, Kentucky
2016	Diplomate, American Board of Orofacial Pain
2016	Conference Travel Award (University of Kentucky) for Society for Neuroscience Annual Meeting, San Diego, California
2016 – 2019	NIH T32 (NIH T32DA016176) Predoctoral Fellowship, University of Kentucky
2012	1st place poster Clinical Sciences - College of Dentistry 2012 Research Day CCTS Spring Conference, Lexington, Kentucky

Peer-reviewed Papers - in revision or preparation

1. **Custodio-Patsey L**, Donahue RR, Fu W, Lambert J, Smith B, Taylor BK. Sex Differences in Kappa Opioid Receptor Inhibition of Latent Postoperative Pain Sensitization in Dorsal Horn". Neuropharmacology.
2. **Custodio-Patsey L,** Taylor BK. Kappa Opioid Receptor Inhibition of AC1 dependent pathways contributes to long-lasting remission of latent sensitization.
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4. Morales Medina J, **Custodio-Patsey L,** Cooper A, Donahue RR, Taylor BK. Supraspinal control of endogenous opioid receptor analgesia at the amygdala in mice.

Published peer-reviewed papers

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