MODULATION OF MULTIPLE MODALITIES OF SOMATOSENSORY INFORMATION BY PERIPHERAL KAPPA OPIOID RECEPTORS

by

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Peripherally-restricted kappa opioid receptor (KOR) agonists are emerging as a novel treatment for pain and itch conditions and have shown efficacy in several recent clinical trials. One primary site of action is thought to be at KORs on primary afferent nerves that innervate the body. Yet, the subtypes of primary afferent somatosensory neurons that express the kappa opioid receptor remain undefined. Using a newly developed *KOR-cre* knockin allele, viral tracing, and single-cell RT-PCR we discovered that that KOR is expressed in a specific subset of peptidergic afferents. This subset targeted multiple tissue types and expressed higher levels of transcripts known to be involved in neurogenic inflammation. Consistent with this, peripherally-restricted KOR agonists inhibited behavioral responses to chemical pain and itch stimuli, but not acute heat stimuli, and also decreased mechanical hypersensitivity following an incision injury. Unexpectedly, we also found that KOR is expressed in subsets of primary afferents that form lanceolate or circumferential endings around hair follicles, suggesting an unappreciated role for KOR signaling in the modulation of low-threshold mechanosensation. At a functional level, genetically-labeled afferents showed inhibition of voltage-gated calcium current in response to kappa agonists, and optogenetic experiments revealed that dynorphin inhibited evoked-EPSCs from the central terminals of KOR-expressing afferents. These experiments provide key insight for the rationale use of peripherally-restricted KOR agonists for the modulation of inflammatory pain, itch, and potentially mechanical allodynia.

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PREFACE

The time I've spent in the Center for Neuroscience program over the past five years has been rewarding in a way that only the most worthwhile challenges can be; these experiences push you but leave you changed for the better, which is an apt description for the work presented here. The thesis work described herein would not have been possible if not for the wonderful, collaborative environment of the Ross laboratory and the Pittsburgh Center for Pain Research that helped shape me as a scientist and made coming to work every day enjoyable. The experiments here cover a breadth of techniques, giving a thoroughness to the investigations that would not have been possible without the assistance of others. I'd like to thank Junichi Hachisuka, Yu Omori, Peter Adelman, Robert Friedman, Margaret Wright, Xiaoyun Cai, Huizhen Huang, Stephanie Fulton, Rick Koerber, and Michael Gold for bringing their insights, talent, and hard work to this project. I'd like to also extend my gratitude to my thesis committee, Drs. Rick Koerber, Michael Gold, Brian Davis, Nathan Urban and my outside examiner Dr. Ben Kolber, who always gave constructive guidance and helped me stay focused. I'm most grateful to my mentor, Dr. Sarah Ross, for her thoughtful leadership, advice, and support. She is someone I admire professionally and personally, and has served as a constant source of inspiration for me. Lastly, I'd like to thank my parents, sister, and husband for their support over the years as they encouraged me through the tough times and helped me celebrate the small victories.

LIST OF COMMON ABBREVIATIONS

KOR – kappa opioid receptor

MOR – mu opioid receptor

DOR – delta opioid receptor

norBNI - Norbinaltorphimine

DRG – dorsal root ganglia

LTMR – low-threshold mechanoreceptor

CGRP – calcitonin gene-related peptide

SP – substance P

TRPV1 – transient receptor potential vallinoid 1

TrkA – receptor tyrosine kinase A

GFRα3 – growth factor receptor alpha 3

Ptgir / PGI_2R – prostacyclin receptor

NF200 – neurofilament 200

TH – tyrosine hydroxylase

IB4 – isolectin B4

IHC – immunohistochemistry

IR – immunoreactive

FISH – fluourescent in situ hybridization

RT-PCR – real time-polymerase chain reaction

GPCR – G protein coupled receptor

VGCC – voltage gated calcium current

AC – adenylyl cyclase

cAMP – cyclic adenosine monophosphate

FLEX.ChR2-tdt – AAV9.CAGGS.FLEX.ChR2-tdtomato.WPRE.SV40

IT – intrathecal

IP – intraperitoneal

IPL – intraplantar

ChR2 – channelrhodopsin 2

EPSC – excitatory post-synaptic current

PWL – paw withdrawal latency

PWT – paw withdrawal threshold

1.0 GENERAL INTRODUCTION

One of the earliest opioid-based treatments for pain was developed by Paracelsus, the 16th century Swiss-German alchemist, who is known today as the father of modern toxicology. Paracelsus referred to this opium-containing tincture as Laudanum, from the latin verb *laudare*, to praise. The name was adopted as a general term for any mixture of opium and alcohol, and gained popularity through use by the English physician Thomas Sydenham in the 1600s. Though a proponent of its use for allaying pain, he warned of the dangers overuse could bring. Even Sydenham himself tried to limit use of the drug in his practices (Davenport-Hines, 2003) likely because he was wary of the very same problems that too often accompany prescription opioid use today.

As Paracelsus and Sydenham observed centuries ago, opioids drugs targeting the mu opioid receptor (MOR) can be potent analgesics and, as such, have been the focus of decades of modern research aimed at developing better treatments for pain. Mu opioid receptor (MOR) agonists, such as morphine, remain among the most widely used treatments for various acute and chronic pain conditions (H. S. Smith & Peppin, 2014). While often effective at relieving pain, MOR agonists are frequently accompanied by deleterious side effects such as respiratory depression, sedation, nausea, constipation, and the development of tolerance (Boom et al., 2012) (Bailey & Connor, 2005). In addition, millions of Americans have been estimated to suffer from substance abuse stemming from mu agonist-based prescription pain relievers due to their high

addictive potential (D. E. Smith, 2017). Yet the class of opioid molecules and their receptors are comprised of several primary subtypes including mu, delta, and kappa (Kieffer & Gaveriaux-Ruff, 2002). As a result, the potential utility of other classes of opioids have been the topic of much interest.

For several decades, the kappa opioid receptor (KOR) has been a promising therapeutic target for pain (for review, see (Millan, 1990; Vanderah, 2010)) and itch (Stull, Lavery, & Yosipovitch, 2016). However, the clinical use of drugs targeting KORs have not been widely adopted. As with MORs, KORs are expressed throughout the body and therefore systemic drugs can have undesired effects that can limit their use. Several approaches to design KOR agonists that specifically target the analgesic and/or antipruritic effects are being investigated, such as biased agonists and peripherally-restricted agonists. Yet if these approaches are to be widely adopted, it is important that we understand what cell types express KOR. This understanding can inform how the opioid system is designed to modulate somatosensory information in naïve conditions, how that changes following an injury or disease, and how best to use pharmacological interventions to ameliorate pain and itch that arise in particular situations.

1.1 KOR MODULATION OF THE SOMATOSENSORY SYSTEM

1.1.1 KOR signaling pathways

The KOR is a member of the seven-transmembrane domain G-protein coupled receptor (GPCR) family. KORs are activated by the endogenous opioid dynorphin, which has a higher binding affinity for KOR over other opioid receptors (Chavkin & Goldstein, 1981); (Chavkin, James, &

Goldstein, 1982); (Huidobro-Toro & Way, 1982). Binding to the KOR by its endogenous ligand, dynorphin, or exogenous ligands, leads to a conformational change and subsequent dissociation of Gi/o coupled Gα and Gβγ subunits.

Opioid receptor activation initiates signaling pathways in which Kir3, the inwardly rectifying potassium channel, is activated through a G protein, pertussis toxin-sensitive mechanism (Al-Hasani & Bruchas, 2011). This effect has been demonstrated in model cell systems (Ikeda, Kobayashi, Ichikawa, Usui, & Kumanishi, 1995); (Henry, Grandy, Lester, Davidson, & Chavkin, 1995); (Ma, Miller, Kuznetsov, & Philipson, 1995); (Ueda, Miyamae, Fukushima, Watanabe, & Misu, 1995); (Ulens, Daenens, & Tytgat, 1999) and in spinal cord neurons (Grudt & Williams, 1993) following application of a KOR agonist. However, direct evidence for a KOR driven effect on Kir3-mediated current in primary afferent neurons is mixed (Marker, Lujan, Loh, & Wickman, 2005); (Fan & Crain, 1995). There are many studies demonstrating that KOR activation inhibits influx of voltage-gated calcium current (VGCC) (Werz & Macdonald, 1984); (Macdonald & Werz, 1986); (Su, Wachtel, & Gebhart, 1998) through high-threshold (N-, P-, and Q-type) voltage-gated calcium channels (Wiley, Moises, Gross, & MacDonald, 1997); (Moises, Rusin, & Macdonald, 1994) by reducing the 'willingness' of channel opening at a given voltage (Bean, 1989); (Gross, Moises, Uhler, & Macdonald, 1990). Although these recordings were performed on the cell body of dissociated dorsal root ganglion (DRG) neurons, these results suggest that KOR activation decreases calcium-dependent neurotransmitter release from axon terminals. In addition to regulating ion channels, KORs also inhibit adenylyl cyclase (AC) and therefore decrease cyclic adenosine monophosphate (cAMP) levels (Grudt & Williams, 1993); (Stein & Zollner, 2009). Inhibition of AC and cAMP production is thought to contribute to the antinociceptive properties of several drugs, including

morphine, as cAMP can increase excitability of sensory neurons through protein kinase A (PKA)-dependent phosphorylation of ion channels such as $Na_{v1.8}$ and TRPV1 (Pierre, Eschenhagen, Geisslinger, & Scholich, 2009). Overall, the effect of KOR activation on sensory neurons is to decrease neuronal excitability.

Following sustained KOR activation, the receptor is phosphorylated by G-protein coupled receptor kinase3 (GRK3) leading to βarrestin-recruitment. Recent studies have shown that this βarrestin bound receptor is not necessarily inactive, as previously thought, and can lead to mitogen-activated protein kinase (MAPK) signaling pathways including ERK ½, JNK 1-3, and p38α, which can impact gene expression and protein phosphorylation. The potential downstream effects of βarrestin-mediated KOR signaling on cell function are still being investigated (Bruchas & Chavkin, 2010).

In summary, the activation of the KOR has been shown to have effects that generally decrease neuronal excitability and neurotransmission, and recent work is starting to investigate how KOR activation of downstream intracellular signaling pathways can affect gene regulation and protein signaling.

1.1.2 Preclinical evidence for KOR involvement in analgesia

Previous pharmacological work has established KOR agonists as having analgesic properties in several preclinical pain models using rodents. One of the earliest, more specific KOR agonists developed, U50,488, has been shown to be analgesic in multiple acute pain assays using either noxious thermal, mechanical, or chemical stimuli (Vonvoigtlander, Lahti, & Ludens, 1983). Systemic administration of non-peptide KOR agonist CI-977 increased mechanical thresholds in the tail clip assay and decreased responses in a model of chemically induced visceral pain

(Hunter et al., 1990). Systemically administered KOR agonist U69,593 decreased thermal sensitivity in the Hargreaves' test (Schepers, Mahoney, Gehrke, & Shippenberg, 2008). These studies show that systemically administered KOR agonists can generate analgesia in multiple assays testing different somatosensory modalities indicating that these drugs may be useful to treat a wide range of clinical pain conditions. As systemically administered KOR agonists likely act on KORs throughout the body, several groups have also investigated the effects of KOR agonist application to specific sites within the CNS. Intrathecal administration of KOR agonists decreased writhing in response to an intraperitoneal (IP) injection of an acidic compound (Porreca, Mosberg, Omnaas, Burks, & Cowan, 1987). Intracerebroventricular, intrathecal administration of the dynorphin analog E-2078 was analgesic in the tail pinch test, tail flick test, and the formalin test (Nakazawa, Furuya, Kaneko, & Yamatsu, 1991). These data suggest that KOR signaling throughout several regions in the CNS, even at the spinal level, has analgesic effects. KOR agonists have also been shown to be analgesic in preclinical models using non-human primates. Dykstra et al. (Dykstra, Gmerek, Winger, & Woods, 1987) reported a decrease in tail withdrawal latency to a noxious thermal stimulus after systemic treatment with U50,488; Sukhtankar et al. (Sukhtankar, Lee, Rice, & Ko, 2014) have reported analgesic effects of systemic U50,488 in an acute thermal assay or in carrageenan-induced thermal hypersensitivity; and Endoh et al. (Endoh et al., 2001) observed an increase in withdrawal latency to a noxious thermal stimulus following systemic nalfurafine administration. These preclinical studies in non-human primates support the translation of KOR agonist-mediated analgesia from rodent models to potential clinical utility in humans.

Pharmacological studies blocking KOR signaling have shown complementary results in preclinical pain assays. Systemic administration of norBNI, a KOR antagonist, aggravated

thermal and mechanical hypersensitivity in the hind paw of mice following an intraplantar injection of complete Freund's adjuvant (CFA) (Schepers, Mahoney, Gehrke, et al., 2008). Systemic, intrathecal, or rostral ventromedial medulla (RVM) injection of norBNI also decreased mechanical thresholds of the non-inflamed paw which suggests the kappa opioid system may have a particularly strong effect on mechanical sensitivity (Schepers, Mahoney, Gehrke, et al., 2008). Intrathecal administration of norBNI prevented the decrease in withdrawal latency in the tail flick assay that is normally observed following a preconditioning noxious thermal stimulus of the hind paw suggesting that KORs at the spinal level are involved in stress-induced analgesia (Yashpal, Pitcher, & Henry, 1995). Intrathecal norBNI also increased flinching in response to intraplantar formalin injection (Ossipov et al., 1996), as did intrathecal pre-treatment with dynorphin antiserum (Ossipov et al., 1996); (Wu, Hung, Mizoguchi, Nagase, & Tseng, 2002). In a model of neuropathic pain, systemic treatment with KOR antagonists norBNI increased mechanical and thermal hyperalgesia observed after nerve injury (M. Xu et al., 2004). These studies using KOR antagonists indicate that the endogenous kappa opioid system is engaged following an injury and acts to mitigate hypersensitivity. In sum, there is substantial evidence that activating KOR or blocking KOR signaling can bidirectionally modulate nociception in preclinical models.

In addition to pharmacological experiments, the role of KOR in antinociception is also supported by studies using KOR knockout (KO) mice. KOR KO mice show increased responsiveness to an intraperitoneal injection of an acidic compound. Yet, KOR KO mice respond similarly to control mice in behavioral models of acute inflammatory, mechanical, and thermal pain. This may be due to compensatory developmental alterations, as the mice used in this study had been lacking KOR expression throughout development. However, the increase in

thermal threshold observed in control mice after administration of KOR agonist U50,488 was not observed in KOR KO mice (Simonin et al., 1998), indicating that KORs are critical for U50,488-mediated antinociception. In a separate study, KOR KO mice showed exaggerated mechanical and thermal hypersensitivity in the CFA model of inflammatory pain (Schepers, Mahoney, & Shippenberg, 2008). Consistent with pharmacological experiments that indicate KOR antagonists are increase nociception, KOR gene knockout mice show exacerbated nociceptive responses, particularly in response chemical stimuli or in an inflammatory state.

Together, pharmacological studies and studies in KOR KO mice provide strong evidence that the kappa opioid system is involved in pain processing, with the dominant effect of KOR activation being antinociception. Although some studies have shown a pronociceptive effect of dynorphin signaling evidence suggests that this is through a non-opioid receptor mediated mechanism (for review see, (Podvin, Yaksh, & Hook, 2016)). The contribution of KORs on specific neuron types or in different regions of the pain pathway and how each might contribute to mediating distinct modalities of pain is less clear. This is important to understand for several reasons. First, to aid our understanding in the context of how the opioid system is organized to regulate somatosensation. Second, to better understand the context for the development of efficacious KOR-based therapies for the treatment of different pain conditions particularly because the changes in the nervous system following different types of injury or disease are unique.

1.1.3 Preclinical evidence for KOR involvement in antipruritus

In addition to a role in nociceptive processing, the kappa opioid system also plays a role in pruritoception. Some of the first connections between the kappa opioid system and itch

processing was reported in 'kappa-dependent' monkeys that had been given multiple injections of KOR agonists. Following withdrawal from KOR agonist injections and subsequent challenge with non-selective opioid receptor antagonist, naloxone, these monkeys showed elevated levels of scratching all over their body (Cowan, 1973); (Gmerek, Dykstra, & Woods, 1987); (Cowan, Kehner, & Inan, 2015). This observation suggests that the kappa opioid system is involved in the tonic suppression of itch sensation. Later, it was observed that KOR agonists decreased scratching behavior caused by the peptide bombesin in rats (Gmerek & Cowan, 1988). Since that time, the KOR agonist nalfurafine (also called TRK-80) has been shown to decrease scratching in preclinical rodent models of acute itch that use intradermal injections of pruritogens that cause scratching through either the histamine-mediated pathway or the non-histamine-mediated pathway (Togashi et al., 2002); (Kardon et al., 2014); (Y. Wang et al., 2005); (Inan & Cowan, 2004); (Umeuchi et al., 2003). Similar antipruritic effects have been observed using the KOR agonist U50,488 (Kardon et al., 2014); (Kamei & Nagase, 2001); (Morgenweck, Frankowski, Prisinzano, Aube, & Bohn, 2015). Nalfurafine has been shown to reduce scratching in preclinical rodent models of chronic itch (Nakao et al., 2008); (Inan & Cowan, 2006); (Kardon et al., 2014). In a rat model of cholestasis, the magnitude of KOR-driven G protein signaling was significantly reduced in the hypothalamus and serum dynorphin levels were lower in cholestatic rats compared to controls, leading the authors to suggest that an imbalance in KOR signaling contributes to cholestatic pruritus (Inan & Cowan, 2005). The modulation of the KOR system has a similar effect on scratching in nonhuman primate models. Nalfurafine and U50,488 have been shown to decrease intravenous morphine-induced scratching (Wakasa et al., 2004), suggesting that these drugs administered systemically can decrease itch sensation. Similar results were observed in monkeys. Following spinal delivery of KOR agonist dynorphin A inhibited β-

endorphin (an endogenous MOR agonist)- and gastrin-releasing peptide (GRP)-induced scratching (Lee & Ko, 2015). Opposite effects are observed with KOR antagonists. When norBNI is injected intradermally into the rostral back, it elicits scratching behavior in mice like that of pruritogen-induced scratching (Kamei & Nagase, 2001). KOR antagonists norBNI and 5'GNTI elicit acute episodes of scratching when injected subcutaneously in the neck area. This effect was decreased in KOR KO mice (Morgenweck et al., 2015) indicating that in wild type mice the endogenous kappa opioid system is regulating pruritoceptive sensitivity. In summary, similar to KOR signaling in analgesia, activation of KOR signaling during pruritus decreases scratching responses, but blocking KOR activation can lead to an elevated pruritoception.

The studies discussed above provide strong evidence that activation of the KOR can induce antipruritus in multiple experimental paradigms. As many of the studies discussed above used systemic administration or constitutive KO models, the contribution of KOR expressed in different cell types or regions of the nervous system cannot be separated which, as discussed below, is important for informing development of kappa opioid-based therapeutics and understanding how the nervous system uses opioids at different points in somatosensory processing.

1.2 STRATEGIES TO IMPROVE UTILITY OF KOR AGONISTS IN A CLINICAL SETTING

While MOR pain relievers' act to decrease both sensory transmission of pain and the affective, unpleasant component of pain, central KORs modulate affect in the opposite direction. KOR agonists can induce conditioned place aversion in rodent models (Zhang, Butelman, Schlussman,

Ho, & Kreek, 2005); (Chefer, Backman, Gigante, & Shippenberg, 2013); Tejeda (Tejeda et al., 2013), and in humans are dysphoric and anxiogenic (Kumor et al., 1986); (Pfeiffer, Brantl, Herz, & Emrich, 1986); (Walsh, Strain, Abreu, & Bigelow, 2001) which is thought to be a result of decreased dopamine release in reward and motivation circuits in the brain (for review, see (Shippenberg, Chefer, Zapata, & Heidbreder, 2001)). KOR agonists can have strong sedative effects (Leighton, Hill, & Hughes, 1988); (Dykstra et al., 1987) and cause diuresis (Leander, 1983); (Blackburn, Borkowski, Friend, & Rance, 1986); (Dykstra et al., 1987). The kappa opioid system plays a central role not only in modulating somatosensory information but also in the regulation of mood, stress, and reward seeking behaviors. Thus, KOR agonists have not been widely used to treat pain and/or itch conditions due to these adverse properties; patients' symptoms may be mitigated, but their emotional states are significantly worsened. In an effort to avoid the dysphoric, anxiogenic, and sedative effects of KOR agonists, two different strategies have been adopted; one of which is to use a biased signaling agonist and the other is to use a peripherally-restricted agonist.

1.2.1 Two main approaches to specifically target KORs for analgesia/antipruritus

Several studies by Chavkin and colleagues have shown using rodent models that p38 MAPK activation via the βarrestin signaling pathway of the KOR is required for the aversive, dysphoric effects of KOR agonists (Bruchas et al., 2007); (Land et al., 2009); (Ehrich et al., 2015), while the G protein signaling pathways underlie analgesic effects (Bruchas et al., 2007). These observations have led to the suggestion that biased, or functionally selective, KOR agonists that do not engage βarrestin may be useful as analgesics without inducing dysphoria (Bruchas & Chavkin, 2010). The Roth laboratory has studied and characterized the bias of many KOR

ligands (White et al., 2014), and has used this information to investigate the signaling pathways underlying various effects of KOR agonists in a preclinical rodent model. Using a strongly G protein biased KOR ligand, RB-64, White et al. (White et al., 2015) observed a reduction in withdrawal latency in the hotplate test and conditioned place aversion to RB-64, but did not observe locomotor deficits or a change in intracranial self-stimulation (ICSS; a model of dysphoria) when the drug was administered systemically. These data support the possibility of using a G protein biased agonists to target analgesic effects of KOR signaling, but RB-64 still caused conditioned place aversion. This study suggests that further investigation of the emotional effects of these types of drugs is warranted, as the effect of biased signaling may vary depending upon the area of the nervous system.

This biased signaling approach has shown promise in preclinical rodent models of itch as well. G protein biased agonists Isoquinolinone 2.1 reduced norBNI induced scratching to a similar extent as U50,488 (which is a very slightly βarrestin biased agonist). U50,488 decreased scratching in both wild-type and βarr2-KO mice suggesting antipruritic effects of KOR agonists may not require βarrestin signaling. (Morgenweck et al., 2015). Further, G-protein biased agonist triazole 1.1 reduced chloroquine-induced scratching and increased withdrawal latency in the tail flick assay without causing sedation in a locomotor assay or suppressing ICSS (an assay of dysphoria) (Brust et al., 2016). Collectively, these data support the idea that analgesic and antipruritic effects of KOR agonists could be accomplished with a G protein biased ligand while possibly mitigating negative psychoactive effects. However, this hypothesis remains to be tested in the clinic.

Another approach to targeting the analgesic and / or antipruritic effects of KOR agonists is to design and administer ligands that do not cross the blood brain barrier (BBB). Targeting

peripheral KOR has the advantage of avoiding activation of CNS KORs that mediate sedative and dysphoric effects. Proof of principle for this approach is evident in reports from several groups have shown that that small volume, site-directed, systemically inactive doses of KOR agonists can have analgesic effects in rodent models of inflammatory pain (Auh & Ro, 2012); (Cunha et al., 2012); (Obara et al., 2009), (Binder et al., 2001); (Keita, Kayser, & Guilbaud, 1995); (Antonijevic, Mousa, Schafer, & Stein, 1995); (Stein, Millan, Shippenberg, Peter, & Herz, 1989). Using systemically administered peripherally-restricted KOR agonists, numerous studies have observed reductions in nociceptive responses in preclinical rodent models. Early studies with the peripherally-restricted KOR agonist asimadoline showed a reduction in pain-related behavior in an adjuvant arthritis model (Binder & Walker, 1998). Several studies using peripherally-restricted KOR agonist ICI204,488 observed efficacy in reducing behavioral responses in acute models of inflammation such as the formalin test (Rogers et al., 1992); (Barber et al., 1994) and the abdominal writhing test (Barber et al., 1994); (Negus, O'Connell, Morrissey, Cheng, & Rice, 2012), as well as in in reducing hypersensitivity in neuropathic pain models (Caram-Salas et al., 2007); (Keita et al., 1995). Studies using tetrapeptide peripherally-restricted KOR agonists (FE200041, FE200665, or FE200666) have shown that these compounds reduce acetic-acid induced writhing, nociceptive responses in the formalin test, acute thermal sensitivity and mechanical hypersensitivity following CFA injection (Vanderah et al., 2004); (Vanderah et al., 2008); (Binder et al., 2001).

Multiple clinical studies have explored the potential of peripherally-restricted KOR agonists for the treatment of pain. Eluxadoline, a mixed MOR / KOR agonist and DOR antagonist that acts primarily in the periphery (Wade et al., 2012), is a recent FDA-approved treatment for irritable bowel syndrome with diarrhea (IBS-D) that is efficacious and has a low abuse potential

(Levy-Cooperman et al., 2016); (Lembo et al., 2016); (Fant, Henningfield, Cash, Dove, & Covington, 2017). Asimadoline, another peripherally-acting KOR agonist, has been shown to be well tolerated in humans, but a Phase III clinical trial with IBS patients was not completed and clinical trials for arthritis treatment were started but did not progress past phase II (Camilleri, 2008); (Mangel & Hicks, 2012); (Barber & Gottschlich, 1997). Enandoline, a peripherally acting KOR agonist, has been used in several clinical trials for the treatment of post-surgical pain but was observed to have use-limiting neuropsychiatric effects at analgesic doses suggesting that at these doses the drug crossed the blood-brain barrier (Pande et al., 1996); (Walsh et al., 2001).

The development of several tetrapeptide, peripherally-restricted KOR agonists have shown promise in clinical trials. Clinical trials from the pharmaceutical company that manufactures CR845, Cara Therapeutics, are testing the therapeutic efficacy of the peripheral KOR agonist in osteoarthritis pain and in various post-operative pain settings. Some of these trials have shown promising results; a significant reduction in pain scores compared to placebo (ClinicalTrials.gov, 2015b); (ClinicalTrials.gov, 2015a). However, the mechanism of a peripherally-restricted KOR agonist-induced reduction in post-operative pain has not been investigated.

Cowan and colleagues have shown that peripherally-restricted KOR agonists can decrease itch sensation in preclinical rodent models (Inan & Cowan, 2004); (Cowan et al., 2015) suggesting that peripheral KOR agonist have effects on multiple somatosensory modalities. Clinically, new therapies for itch are advancing rapidly, one of which is the above mentioned KOR agonist nalfurafine (also known as TRK-80). Nalfurafine is clinically approved in Japan to treat uremic pruritus and cholestatic pruritus, and is currently in phase 2 clinical trials to treat prurigo nodularis, a condition characterized by pruritic nodules on the skin. Butorphanol, a mixed KOR agonist/MOR antagonist, has also shown efficacy at reducing itch in patients with

intractable pruritus. Both of these drugs penetrate to the CNS, and there are some side effects associated with butorphanol treatment, such as nausea, drowsiness, and abnormal dreams. Nalfurafine seems to be well tolerated (Stull et al., 2016) but the underlying mechanism as to why is unclear. There are ongoing clinical trials using peripherally-restricted KOR agonists CR-845 and asimadoline to test their efficacy in treating pruritus (ClinicalTrials.gov, 2016b); (ClinicalTrials.gov, 2016a).

Overall, there is substantial evidence from preclinical and clinical studies for kappa opioid regulation of pain and itch sensation via KORs in various regions of the nervous system, including the periphery. Although there is evidence for expression of KORs on primary afferent neurons, there is also some evidence for expression on other peripheral cell types (Cai et al., 2016); (Wittert, Hope, & Pyle, 1996); (Gaveriaux, Peluso, Simonin, Laforet, & Kieffer, 1995). Therefore, the characterization of these neurons and other cell types and the effect that KOR activation has on their activity is importamt for determining the mechanisms underlying peripherally-restricted KOR agonist-induced analgesia and antipruritus. This knowledge can aid in the development of targeted treatments for specific pain and itch conditions.

1.3 KOR EXPRESSION IN PRIMARY AFFERENTS

As discussed above, preclinical and clinical studies have established a role for KORs in modulating nociception and pruritoception, even by KOR drugs that do not cross the BBB and are restricted to the periphery. For drugs targeting KORs to be used as effective therapies, it is important to characterize the primary afferents expressing KOR to best understand how this system modulates somatosensation as these neurons transmit somatosensory information to the

CNS. Previous work has established *Opkr1* mRNA and KOR protein expression in primary afferents in the DRG. *In-situ* hybridization (ISH) studies investigating opioid receptor mRNA distribution in primary afferent populations in the DRG report that *Oprk1* mRNA is predominantly found in small-to-medium diameter afferents (Mansour et al., 1994); (Mansour, Burke, Pavlic, Akil, & Watson, 1996); (M. K. Schafer, Bette, Romeo, Schwaeble, & Weihe, 1994) which is consistent with expression on putative nociceptors, although there was mention of faint labeling in larger diameter neurons (Mansour et al., 1996) which are not necessarily nociceptive neurons. An immunohistochemical (IHC) study using a KOR antibody reported similar findings of KOR protein expression mainly in small diameter neurons; about 9% of the total number of DRG afferents in naïve rats were immunoreactive (IR) for the KOR (Ji et al., 1995). To characterize these afferents in more detail a few colocalization studies have been performed. These studies have found overlap between *Tac1* mRNA (encoding the neuropeptide substance P) and *Oprk1* mRNA (Minami, Maekawa, Yabuuchi, & Satoh, 1995), and a study in acutely cultured rat DRG neurons observed KOR-immunoreactivity in afferent subtypes that also express the neuropeptides CGRP and/or substance P (Rau, Caudle, Cooper, & Johnson, 2005). These data suggest that KOR is expressed on at least of subgroup of peptidergic primary afferents, many of which are nociceptors and likely involved in neurogenic inflammation (Grant, Gerard, & Brain, 2002). Physiological recordings of DRG neurons in culture have shown that some afferents that respond to multiple opioid receptor agonists, but responsiveness to one opioid receptor does not necessarily mean an afferent responds to another or all agonists, suggesting that there are distinct subtypes of primary afferents expressing different opioid receptor types (Werz & Macdonald, 1984); (Macdonald & Werz, 1986); (Moises et al., 1994). Together, these studies consistently report KOR expression in small diameter, putative

nociceptive neurons which aligns with reports that KOR agonists, particularly peripherally-restricted agonists, dampen nociceptive and pruritoceptive processing. Yet detailed characterization of the primary afferent subtypes expressing KOR, beyond overlap with other opioid receptor and neuropeptides, has not been reported. This information would increase our understanding of how KORs on primary afferent neurons act to modulate somatosensory information.

1.3.1 Localization of KOR on central and peripheral terminals

Primary afferent neurons are pseudo-unipolar neurons and have endings in both the CNS that terminate in the dorsal horn of the spinal cord and endings in the PNS that terminate in various peripheral tissues (Kandel, 2012). KORs can modulate transmission through primary afferent neurons via expression at either terminal. Although peripherally-restricted KOR agonists are a popular target in terms of drug development, untangling the effect of KOR activation on the circuitry in the spinal cord is important as well. If peripherally-restricted KOR agonists are not successful in clinical trials, an alternative method of targeting KOR for therapeutic use could be spinal delivery via an epidural injection. There is supportive evidence in the literature that kappa opioids play a role in modulating somatosensory circuitry in the dorsal horn (Randic, Cheng, & Kojic, 1995) (Kardon et al., 2014), but there are still some uncertainties remaining regarding KORs' role on primary afferent terminals.

Several groups have identified and investigated the function of dynorphin-expressing spinal dorsal horn interneurons and report findings consistent with these interneurons regulating itch sensation or mechanical pain after injury (Sardella et al., 2011); (Kardon et al., 2014); (Duan et al., 2014). These neurons consist predominantly of inhibitory, GABA and / or glycinergic

interneurons, but there is also a smaller population of excitatory, glutamatergic dynorphin-expressing neurons (Sardella et al., 2011). There is a likelihood that, in addition to acting on KORs expressed on spinal neurons, dynorphin also acts on KORs expressed on the central terminals of primary afferents. MOR and DOR agonists have been shown to inhibit glutamate release from the central terminals of sensory afferents (Francois et al., 2017; Heinke, Gingl, & Sandkuhler, 2011; Kohno, Kumamoto, Higashi, Shimoji, & Yoshimura, 1999). There is some evidence that KOR agonists may likewise inhibit primary afferents in superficial lamina (Randic et al., 1995), but this effect has not been universally observed (Ikoma et al., 2007; Kohno et al., 1999).

KOR antibody staining has been observed in peripheral nerve fibers (Labuz et al., 2009) and accumulation of KOR protein at the proximal site of a ligated sciatic nerve has been inferred from increased intensity of KOR-IR in this area, suggesting that the KOR is trafficked to the periphery (Ji et al., 1995). This suggests that KOR protein is translated at the soma and transported to peripheral terminals. Several studies have reported results that support the presence of KOR on peripheral nerve terminals. For example, application of KOR agonists to a peripheral site of stimulation either decreases nerve activity or neuropeptide release (Averbeck, Reeh, & Michaelis, 2001); (Russell, Leslie, Su, Watkins, & Chang, 1987); (Andreev, Urban, & Dray, 1994). In summary, these data suggest that KOR protein is transported to the central and peripheral terminals and can play a functional role in modulating somatosensory transmission at each level.

1.4 GAP IN KNOWLEDGE

Although preclinical studies using peripherally-restricted KOR agonists have shown reductions in pain- and itch-related behaviors, the move from animal models to clinical trials in humans is accompanied by many complexities that cannot be modeled in the lab. For clinical trials to be effective, a targeted approach based on a mechanistic understanding on the kappa opioid system may have the highest likelihood of succeeding. Therefore, understanding the primary afferent types that express the KOR and how activation of this receptor affects nociceptive and pruritoceptive processing is important if peripherally-restricted compounds are to be further developed and used successfully for the treatment of pain and itch conditions.

An interest in defining distinct neuronal types has been a large driver of our understanding of how the nervous system is organized to process information since the early drawings of Ramon y Cajal at the turn of the 20th century. Neuronal anatomy is a critical aspect of cell type definition, as the structure of neuronal types and their connectivity to other regions give insight into their functional properties. More modern techniques have allowed for cell type definition through physiological properties, protein expression, and even wide-scale transcriptional profiling. The growing use of genetic mouse models has given neuroscientists the ability to visualize neuronal types of interest, to characterize their anatomical structure and connectivity to other neuronal types in order to inform circuit function. These models also allow for the expression of exogenous proteins in specific neuronal types, allowing researchers to manipulate their activity; such as the use of light-gated channels to excite or inhibit neuronal activity within a particular circuit and / or during a particular behavior (Deisseroth, 2015). This approach has led to advances in our understanding of the role of different neuronal cell types in

disorders such as schizophrenia (Cho & Sohal, 2014) and Parkinson's disease (Vazey & Aston-Jones, 2013).

Primary sensory afferents in the DRG are no exception to the interest in the definition of neuronal cell types, which could inform not only how the nervous system processes different somatosensory stimuli, but also lead to insights for the treatment of disease. Traditionally, primary afferent neuron subgroups were differentiated based on the characteristics of soma size, conduction velocity, anatomy of their peripheral terminals, and the lamina they terminate in the dorsal horn (Le Pichon & Chesler, 2014); (Kandel, 2012). Four primary afferent fiber types – C, Aδ, Aβ, and proprioceptors – are each generally involved in different roles in somatosensory processing. However, there is a depth of diversity within primary afferents that is not fully captured by these classifications or the characteristics they are based on. Molecular genetic techniques have allowed for the further division of subgroups and the study of their function (Le Pichon & Chesler, 2014). Other groups have built upon this understanding by using single cell transcriptional profiling in an effort to identify functional groups based on unbiased classification schemes (Usoskin et al., 2015); (C. L. Li et al., 2016); (Chiu et al., 2014). However, the opioid receptors have not been robustly detected in these approaches and hence it is unclear which of these *de novo* subgroups of DRG neurons express the KOR, or if KOR-expressing afferents are a new subgroup of their own. Understanding which functional neuronal subtypes express KORs may lead to new advancements in the understanding of opioid regulation in primary afferents, and tools that allow for the identification and manipulation of this subgroup can advance our knowledge of their involvement in somatosensory processing.

Given the strong evidence for regulation of nociception and pruritoception through kappa opioid signaling, the need for an alternative strategy to specifically harness the analgesic and/or

antipruritic properties of KOR agonists, evidence for the expression of KOR on primary afferent terminals in the periphery, and the ability of KORs to affect the processing of these cells, our goal was to better characterize the expression pattern of KOR in primary afferents and understand the role of KOR on in modulating primary afferent activity. The experiments herein aim to characterize the primary afferents subtypes expressing the KOR and to use this information to perform studies investigating the effect of KOR activation on somatosensory transmission at the level of the periphery.

2.0 KOR IS EXPRESSED BY AT LEAST TWO DISTINCT GROUPS OF PRIMARY AFFERENTS

The work described within this chapter is part of a manuscript that is under review. The draft of manuscript was prepared by myself and Dr. Sarah Ross, with review and editing by Drs. Rick Koerber and Michael Gold. FISH experiments were performed by Elizabeth Sypeck and Dr. Gregory Scherrer. Recordings from dissociated DRG neurons were conducted by myself with guidance from Dr. Michael Gold. IHC experiments were conducted, imaged and analyzed by myself, with assistance of IT injections from Yu Omori and intrasciatic injections from Zeyu Hu. Dr. Peter Adelman conducted experiments and analyzed the data for the teased fiber recordings. Drs. Peter Adelman and Margaret Wright performed the backlabeling, dissociation and single cell pick-up for the single cell RT-PCR experiments. All primers were designed by Robert Friedman, who also conducted all RT-PCR experiments. Analysis of the single-cell RT-PCR data was performed by myself with the assistance of Excel spreadsheets designed by Robert Friedman and Dr. Peter Adelman. Figures were prepared and drafted by myself with editing by Dr. Sarah Ross.

2.1 INTRODUCTION

As we are particularly interested in how the nervous system processes somatosensory information and in developing better treatments for pain and itch conditions, the first cell type(s) expressing KOR we wanted to characterize were primary sensory afferents in the DRG. Previous work has reported that KOR is expressed primarily in small-to-medium diameter neurons through characterization of the primary afferent types that express KOR in the DRG using either ISH or IHC (Ji et al., 1995); (Mansour et al., 1994); (Maekawa et al., 1994); (M. K. Schafer et al., 1994), but more extensive characterizations of the afferents expressing KOR have been limited to co-expression of one or two other proteins (Ji et al., 1995); (Minami et al., 1995); (Rau et al., 2005) or responsiveness to one other agonist (Werz, Grega, & MacDonald, 1987); (Moises et al., 1994).

In somatosensory research, there has been a recent and growing interest in characterizing and defining subgroups of primary afferents within the DRG. Several recent papers have described analyses of transcripts on a single cell level, as unique transcript expression profiles may lead to novel definitions of cell types and may provide new insights into functional properties of sensory afferents (Usoskin et al., 2015; Li et al., 2016; Chiu et al., 2014). Yet, opioid receptor transcripts have not been robustly detected in analyses of single-cell RNA sequencing of sensory afferents (Usoskin et al., 2015); (C. L. Li et al., 2016); (Chiu et al., 2014). Thus, a reliable tool to identify KOR-expressing neurons is much needed to progress our understanding of the characteristics of these afferents, the role of KOR signaling in somatosensation, and how best to target this receptor for therapeutic use. Having a tool that can reliably identify cells that express the KOR would go a long way towards increasing our ability to study how kappa opioid signaling effects cellular processing (including somatosensation) and how drugs targeting this receptor could be

optimally designed and delivered to achieve a desired effect. Often, progress towards characterizing a cell type that expresses a particular protein is made by using an antibody that specifically recognizes the protein marker of interest. However, an antibody that produces reliable staining of the KOR has been notoriously difficult to find.

Previously, Cai et al. (Cai et al., 2016) reported the generation of a *KOR-cre* knockin allele. When *KOR-cre* cells were visualized using a $Rosa^{lsl\text{-}tdt}$ allele, cells throughout the nervous system were labeled, including primary afferent neurons in the DRG. Therefore, the experiments herein aimed to determine if the *KOR-cre* allele could be used to reliably identify primary afferents expressing KOR. If that was the case, this tool could be used to characterize the neurochemical, anatomical, and transcriptional aspects of KOR-expressing neurons. This information would then form the basis for the design of studies investigating the effects of KOR agonists on somatosensory processing. Based on the previous literature investigating the distribution of KOR in the DRG, their functional properties, and the effectiveness of KOR agonists in reducing various forms of nociception and pruritoception, it was predicted that KOR would be expressed primarily by small diameter, peptidergic, nociceptive / pruritoceptive neurons that target skin in addition to deeper tissues of the body.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Mice were given free access to food and water and housed under standard laboratory conditions. The use of animals was approved by the Institutional Animal Care and Use Committee of the

University of Pittsburgh. KOR-cre knockin mice were generated by targeted Cre recombinase into the endogenous KOR locus (*Oprk1*) as previously described (Cai et al., 2016) and are maintained on a mixed C57bl/6.129J background. For electrophysiology, immunohistochemistry, and single cell RT-PCR experiments, KOR-cre heterozygous mice were mated with Ai9 cre-responsive tdTomato reporter mice (Madisen et al., 2010) or with Ai32 cre-responsive ChR2-eYFP reporter mice (Madisen et al., 2012)(The Jackson Laboratory).

2.2.2 Electrophysiology

Patch-clamp recordings in dissociated DRG neurons

Mice (5-8 weeks old) were deeply anesthetized and transcardially perfused with ice-cold Ca2+/Mg2+-free HBSS (Invitrogen). Bilateral DRG (L2–L5) were dissected into cold HBSS and dissociated as described previously (S. A. Malin, Davis, & Molliver, 2007). Cells were plated in DMEM F-12 (Invitrogen) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 50 U/ml). Two hours later, coverslips were flooded with Ca2+/Mg2+-free HBSS (Invitrogen) containing 5 mM HEPES and stored at room temperature. Experiments were performed within 8 h of tissue harvest.

Borosilicate glass electrodes were filled with (in mM) 100 Cs-methanesulfonate, 5 Na-methanesulfonate, 40 TEA-Cl, 1 CaCl2, 2 MgCl2, 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP, pH 7.2 (adjusted with Tris-base), 310 mOsm (adjusted with sucrose). Neurons were continuously superfused with a bath solution that contained (in mM) 100 Choline-Cl, 30 TEA-Cl, 2.5 CaCl2, 0.6 MgCl2, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with Tris-base), 320 mOsm (adjusted with sucrose). Currents were evoked up to three times from each neuron before, during, and after drug application with the following voltage-clamp protocol: 50ms, 70mV steps

from a holding potential of −70mV to 0mV. Series resistance compensation (>70%) was used for all voltage-clamp recordings. To reduce the impact of leak currents, a p/4 leak subtraction protocol was used with a holding potential of −90 mV during the acquisition of the leak pulses. Response to drug application was defined as at least a 10% change from baseline.

Extracellular single-fiber recordings

Recordings were performed as previously described (McIlwrath, Lawson, Anderson, Albers, & Koerber, 2007). Briefly, Mice (4 - 6 weeks old) were deeply anesthetized, the hindlimb was shaved, and mice were perfused transcardially with oxygenated 6 °C sucrose-based artificial cerebral spinal fluid (ACSF) in mM: 39.0 sucrose, 0.9 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26.0 $NaHCO_3$, 10.0 D-glucose. The saphenous nerve and its innervation field were excised, transferred to a dual chamber recording dish, superfused with chilled oxygenated synthetic interstitial fluid (SIF) in mm: 123.0 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 1.7 NaH_2PO_4, 2.0 $CaCl_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose, 10 HEPES, pH 7.4, and warmed slowly to 31 °C. The skin was pinned epidermal-side up on a rigid perforated metal platform, and the level of the circulating SIF was adjusted to bathe the corium side from underneath. This allowed for mechanical and optical stimulation of the dry skin (adapted from (Koerber & Woodbury, 2002)). The saphenous nerve was threaded into a mineral oil-filled recording chamber and placed on top of a small mirror for dissection. The nerve was de-sheathed and extracellular recordings were obtained from functionally identified single myelinated fibers in teased filaments draped over a silver hook electrode (Kress, Koltzenburg, Reeh, & Handwerker, 1992; Reeh, 1986). Signals were amplified, digitized, and recorded using Spike2 software for offline analysis.

Once an individual fiber was isolated, its receptive field (RF) was located using a small brush and tested for light responsiveness using a blue laser (473 nm). Conduction velocity was

assessed using a concentric electrode in the RF (distance to electrode/stimulus-response delay). Myelinated fibers were defined as conducting faster than 1 m/s (Koltzenburg, Stucky, & Lewin, 1997). A feedback-controlled constant force stimulator (Dual Mode Lever System, Aurora Scientific, Aurora) with a 1-mm diameter contact area was used to determine mechanical threshold of the cell and to characterize its response to suprathreshold stimuli. Constant force stimuli consisting of square waves of 5 sec duration were applied in an ascending series of 1, 5, 10, 25, 50, and 100 mN with 30-s interstimulus intervals.

2.2.3 Intrathecal (IT) injections

Intrathecal injections were performed as previously described (Kardon et al., 2014). Mice were anesthetized (2.0% for induction and 1.5% for maintenance) with isoflurane in a flow of O2, placed in a prone position on top of a heating pad covered with a blue fiber disposable towel, and the hair on their back was clipped. The caudal paralumbar region was securely held by the thumb and middle fingers of the left hand, and the index finger was placed on the tip of sixth lumbar (L6) spinous process, the highest point of the vertebral column. All intrathecal injections were delivered in a total volume of 5 μL using a 30-gauge needle attached to a luer-tip 25 μL Hamilton syringe. The needle was inserted into the tissue at a 45° angle and through the fifth intervertebral space (L5–L6) causing a sudden lateral movement of the tail. Solution was injected at a rate of 1 μL/s. The needle was held in position for 10 s and removed slowly to avoid any outflow of the solution. Anesthesia was discontinued and the mice recovered from anesthesia within 5 min.

2.2.4 Histology

For immunostaining of tissue sections, 20µm thick sections were cut with a cryostat (Leica) and mounted onto Superfrost Plus slides (Fisher Scientific) and stored at -20°C until use. Tissue sections were blocked in 10% donkey serum and 0.25% Triton-X in PBS for 1 h at room temperature. Sections were incubated with primary antibodies in block overnight at 4-8°C. Slides were washed 4 x 5 min in PBS containing 0.1% triton-X. Primary antibodies were revealed with species-specific secondary antibodies raised in donkey and conjugated to Pacific Blue, Alexa488, Alexa555, or Alexa647 (Life Technologies) diluted at 1:500 in block, incubated for 1 hour at RT, washed as above, and coverslipped. The primary antibodies used were: sheep anti-CGRP (1:2000, Abcam, ab22560), rabbit anti-NF200 (1:1000, Sigma Aldrich, N4142), mouse anti-NF200 (1:500, Sigma Aldrich, N0142), rabbit anti-TH (1:1000, Millipore, AB152), mouse anti-parvalbumin (1:1000, Millipore, MAB1572), rabbit anti-PGP9.5 (1:1000, UltraClone Limited, RA95101), rabbit anti-substance P (1:10000, Immunostar, 20064), goat anti-TRPV1 (1:1000, Santa Cruz Biotechnologies, sc-12489), rabbit anti-RFP (1:1000, Rockland, 600-401-379S), and rabbit anti-TRPM8 (1:500, gift from M. Tominaga, Okazaki Institute for Integrative Bioscience). IB4 binding was visualized using biotinylated IB4 (Sigma, 1:500, L2140) and fluorophore-conjugated streptavidin (Life Technologies, 1:500) in place of primary and secondary antibodies.

For wholemount immunostaining of hairy skin, the hair was shaved using clippers following perfusion, and back skin was dissected. Skin sections were post-fixed for approximately 30 minutes in 4% PFA then rinsed in 1X PBS. Underlying adipose tissue was dissected from the skin, and then tissue was cut into small pieces, approximately 1 cm x 0.5 cm. Tissue was rinsed 30 min x 5 – 8 hours at room temperature in PBS containing 0.3% triton-X,

with each vial containing one tissue square and at least 1mL volume. Tissue was incubated in primary antibodies (listed above) diluted in PBS containing 0.3% triton-X, 5% normal donkey serum, and 20% dimethylsulfoxide (DMSO) for 4 – 8 days at 4-8°C with agitation. Tissue was then washed as above. Primary antibodies were revealed with species-specific secondary antibodies raised in donkey and conjugated to Alexa488, Alexa555, or Alexa647 (Life Technologies) diluted at 1:500 in block, incubated for 2 – 4 days at RT with agitation. Tissue was washed as above, mounted on slides and coverslipped.

Slides were imaged using a Nikon A1R confocal microscope through either a 20x or 60x oil-immersion lens. Full-tissue thickness z-stacks (0.5-1 μm sections) were taken. For quantification of co-localization of KOR-cre positive neurons, images were analyzed and neurons were counted using Nikon Elements Advanced Research Software.

For quantification of co-localization of *KOR-cre* positive neurons, full-tissue thickness z-stack images of lumbar (L1 - L6) DRGs were analyzed. At least seven individual sections DRG were analyzed per mouse for each marker analyzed, and at least 3 mice were analyzed per developmental time point. Sections of DRG analyzed were at least 100 μm apart in order to avoid counting a single neuron multiple times, and only neurons with visible nuclei were counted. Neurons were counted using Nikon Elements Advanced Research Software.

2.2.5 Single-cell RT-PCR

Backlabeling

Mice were anesthetized with isoflurane (2%) and an incision was made on the medial surface of the right thigh, near the knee. The saphenous nerve was isolated with an effort to cause only a minimum amount of soft tissue damage and the nerve was insulated from the surrounding tissues

using parafilm. The chosen dye (1% Alexa 488-wheat germ agglutinin (WGA)), was injected into the nerve via a quartz pipette using a picospritzer (World Valve Corporation, Picospritzer II). The area was rinsed with saline, the parafilm was removed, and the wound was closed using silk sutures.

Cell dissociation and pickup

Mice (5-8 weeks old) were deeply anesthetized and transcardially perfused with ice-cold Ca2+/Mg2+-free HBSS (Invitrogen). Bilateral DRG (L2–L5) were dissected into cold HBSS and dissociated as previously described (Malin et al., 2007). Cells were plated onto laminin coated coverslips and then placed in culture conditions for 45 minutes to promote adhesion. Before pickup, cultures were flooded with cell collection buffer (in mM) 140 NaCl, 10 glucose, 10 HEPES, 5 KCl, 2 CaCl2, 1 MgCl2). Fluorescent cells were picked up with borosilicate glass pipettes (World Precision Instruments) held by a 3-axis micromanipulator. After confirming pickup using fluorescence, pipette tips were broken off into tubes containing 3uL of lysis buffer (Epicentre, MessageBOOSTER kit), and stored at -80^{o}C until use.

Single cell amplification and qPCR

Transcripts from single cells were reverse transcribed and linearly preamplified using the MessageBOOSTER kit for cell lysate (Epicentre). After preamplification, the products were cleaned with RNA Cleaner & Concentrator-5 columns (Zymo Research) and transcript levels were quantified using qPCR with optimized primers and SsoAdvanced SYBR Green Master Mix (BioRad). Cycle-time (Ct) values were determined using regression. Quantification threshold was determined to be interreplicate average of 35 Ct, the point where replicates have a 95% chance of reoccurring, and the GAPDH threshold for cell inclusion was set to 25 Ct to ensure we could detect transcripts a thousand-times less prevalent than GAPDH. If a cell met the criteria for

inclusion, it would be corrected for primer efficiencies using the Pfaffl method (Pfaffl, Lange, Daxenberger, & Meyer, 2001).

2.3 RESULTS

2.3.1 *KOR-cre* knockin mouse as a tool to identify primary afferents expressing KOR

In order to identify cells that express the KOR, a *KOR-cre* knockin allele was used where cre recombinase (cre) replaces the initial coding sequence of the *Opkr1* gene (Cai et al., 2016). Crossing these mice to mice harboring an allele with a cre-dependent fluorescent reporter (the $Rosa^{lsl\text{-}tdtomato}$ allele, known as Ai9; Jax labs) allows us to visualize *KOR-cre* $Rosa^{lsl\text{-}tdtomato}$ primary afferents in the offspring. However, this approach results in tdtomato (tdt) expression in cells that have expressed *KOR-cre* during any point of development and therefore, tdt expression is not necessarily representative of neurons that express KOR protein in adulthood. So as to target the neurons expressing *KOR-cre* in the DRG during adulthood, we performed an intrathecal (IT) injection in adult *KOR-cre* mice (P39-P40) of cre-dependent virus AAV9.CAGGS.FLEX.ChR2-tdt.WPRE.SV40 (FLEX.ChR2-tdt; PennVector) (Vulchanova et al., 2010); (Dayton, Wang, & Klein, 2012). This approach is intended to target expression of the light-gated ion channel channelrhodopsin 2 (ChR2) fused to tdt, but only in neurons expressing cre, and therefore also highly likely expressing KOR, at the time of injection.

First, in collaboration the Scherrer laboratory, dual fluorescent *in-situ* hybridization (FISH) was conducted to determine the overlap of *Oprk1* mRNA and *FLEX.ChR2-tdt* expression (Figure 1A). The vast majority of the *FLEX.ChR2-tdt* positive neurons co-localized with *Oprk1*

expression (92.33% ± 3.70%; n=3 mice), and 93.62% ± 2.90% (n=3 mice) of the *Oprk1* expressing neurons were co-localized with *FLEX.ChR2-tdt* (Figure 1A-B). Second, in collaboration with the Koerber laboratory, to verify that the *KOR-cre* allele is specific to neurons that express KOR, single-cell RT-PCR on FLEX.ChR2-tdt positive neurons was performed. We found that 83% (10/12 cells) of these cells expressed detectable levels of Oprk1 mRNA (Figure 1C). In comparison, we found that no tdt negative DRG neurons from *KOR-cre Rosa*$^{lsl\text{-}tdt}$ mice expressed detectable levels of Oprk1 mRNA (0/24 cells; 8 shown for illustrative purposes) (Figure 1C). We chose tdt negative cells from *KOR-cre Rosa*$^{lsl\text{-}tdt}$ mice to ensure that, in using the *KOR-cre* allele, we were not neglecting to analyze a major population of DRG neurons that actually do express KOR but fail to cause recombination via *KOR-cre*. Together, these results show that using viral techniques to infect primary afferent neurons in adult *KOR-cre* mice is sufficient to cause recombination in primary afferents that are highly likely to express KOR, as the majority express *Oprk1* mRNA, and that this approach is specific as cells that do not show recombination with the *KOR-cre* allele do not express *Oprk1* mRNA.

Previous studies in dissociated DRG neurons have demonstrated that application of a KOR agonist can cause inhibition of voltage-gated calcium current (VGCC) in a subset of primary afferent neurons (Bean, 1989); (Macdonald & Werz, 1986); (Moises et al., 1994); (Su et al., 1998); (Wiley et al., 1997). Therefore, we performed whole-cell patch clamp recordings on acutely dissociated DRG neurons, and measured the effects of dynorphin on VGCC to determine if *KOR-cre* neurons express functional levels of KOR protein. When recording from tdt-positive cells that were genetically labeled by the *KOR-cre* allele, dynorphin (0.1 or 1 μM) caused a significant decrease in VGCC in 77% of *KOR-cre* neurons (17/22 cells) (Figures 1D, 1E, and 2B). In many cells VGCC amplitude returned to baseline upon washout of dynorphin (Figure

2A). Dynorphin application was also associated with a significant increase in rise time (Figure 2C), consistent with the possibility that the inhibition of peak inward current reflects a KOR-induced rightward shift in the voltage-dependence of channel activation (Bean, 1989). Moreover, the effects were specific to KOR since the decrease in current caused by dynorphin was blocked by the KOR antagonist nor-BNI (Figures 1D, 1E and 2D). Primary afferents that were not genetically labeled by the *KOR-cre* allele showed neither a decrease in the magnitude of VGCC nor an increase in rise time upon application of dynorphin (Figures 1E, 2B and 2C). These findings suggest that the *KOR-cre* allele causes recombination in primary afferents that express functional KOR.

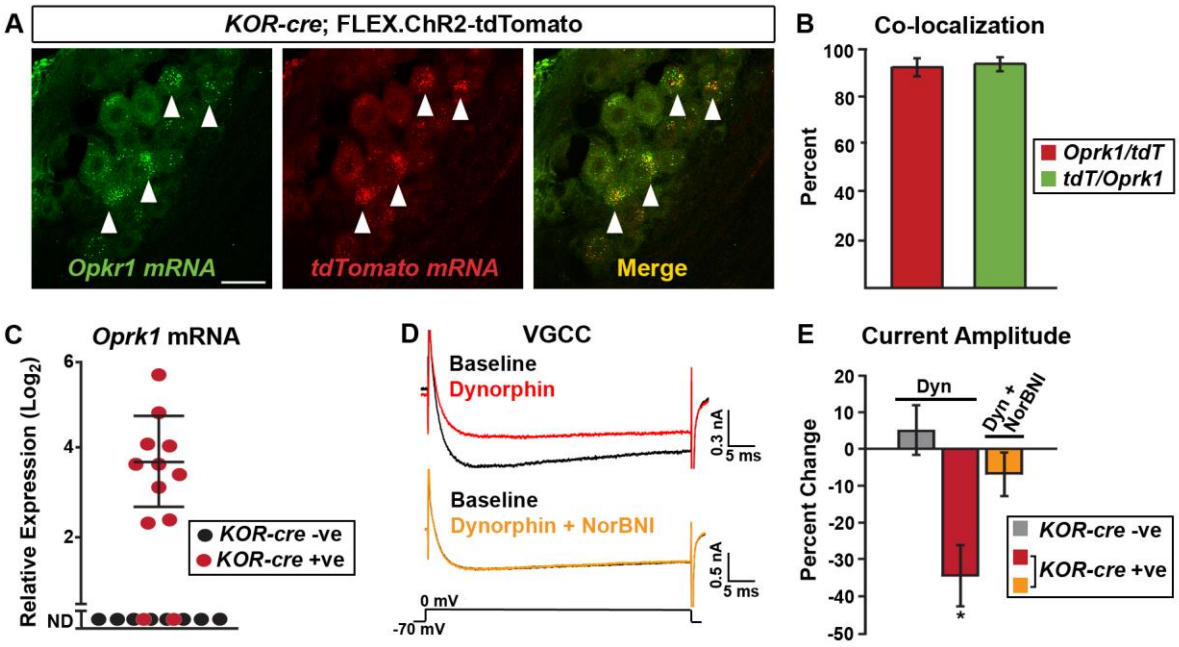


Figure 1. *KOR-cre* as a tool for targeting KOR-expressing DRG neurons

A. Dual FISH of *Oprk1* (green) and *tdTomato* mRNA (red) shows high co-expression (merge) in DRG neurons. Lumbar DRG neurons were infected with the Cre-dependent virus (AAV.FLEX.ChR2-tdTomato) via IT injection at P40. Arrowheads indicate cells co-expressing *Oprk1* and *tdTomato* mRNA. Scale bar = 25 µm.

B. Quantification of (A). Most *tdTomato* positive neurons (red) co-expressed *Oprk1* mRNA, and most *Oprk1* postitive neurons (green) co-expressed *tdTomato.* n = 3 mice. Data are presented as mean ± SEM.

C. Single-cell RT-PCR of lumbar DRG neurons. *KOR-cre* mice were infected with an AAV.FLEX.ChR2-tdTomato virus via IT injection at P40. The majority of *KOR-cre;* AAV.FLEX.ChR2-tdtomato positive neurons, (red dots) express detectable levels of *Oprk1* mRNA (8 of 10), while none (0 of 22) of the *KOR-cre* negative neurons (black dots) express detectable levels of *Oprk1* mRNA (only 8 cells are shown for clarity; ND, not detected). Data are presented as the $-\log_2 \Delta CT$ expression relative to *GAPDH* expression within the same cell such that bigger numbers represent higher mRNA expression. Dots represent data points from individual cells.

D. Representative traces of voltage-gated calcium currents (VGCC) in a genetically-labeled cell from a *KOR-cre; Rosa26lsl-tdTomato* mouse at baseline (black traces, top and bottom), in the presence of dynorphin (1 µm, red; top trace), and in the presence of dynorphin (1 µm) + norBNI (1 µm, yellow; bottom trace). Neurons were held at -70 mV and a 50 ms step to 0 mV was applied.

E. Quantification of the percent change in current amplitude of *KOR-cre* negative neurons (gray; n = 5), *KOR-cre* positive neurons in the presence of dynorphin (1 µm; red; n = 6), or *KOR-cre* positive neurons in the presence of dynorphin (1 µm) + norBNI (1 µm; orange; n = 3). There was a significant decrease in current amplitude in the presence of dynorphin in *KOR-cre* positive neurons (*, paired t-test, $p < 0.05$). Data are presented as mean ± SEM.

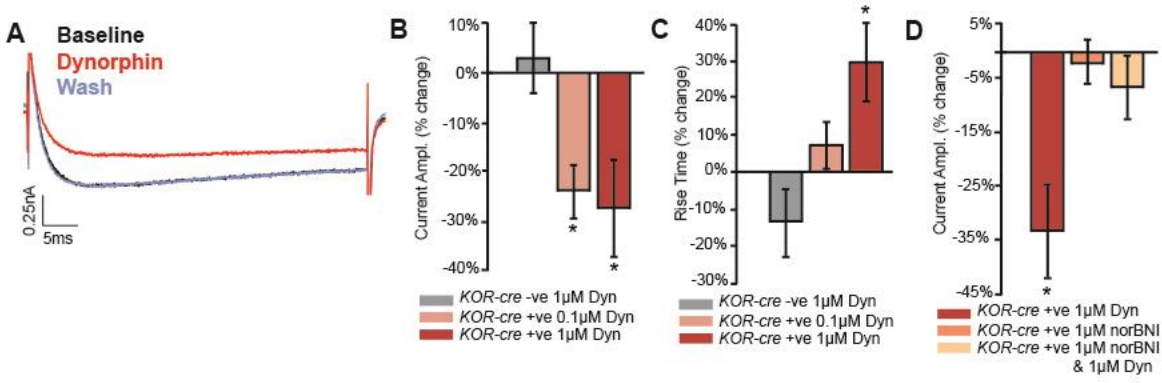


Figure 2. The inhibition of VGCC by dynorphin is blocked by the opioid receptor antagonist norBNI.

A. Representative traces of VGCC in a *KOR-cre*-positive neuron at baseline (black), in the presence of dynorphin (1 µm; red), and after washout (blue). Neurons were held at -70 mV and a 50 ms step to 0 mV was applied.

B - C. Quantification of the percent change in current amplitude (B) or rise time (C) of either *KOR-cre* negative neurons (gray; n = 5 neurons), *KOR-cre* positive neurons in the presence of 0.1 μm dynorphin (pink; n = 7 neurons), or of *KOR-cre* positive neurons in the presence of 1 μm dynorphin (red; n = 6 neurons). There was a significant decrease in current amplitude in the presence of either 0.1 or 1 μm dynorphin in *KOR-cre* positive neurons relative to baseline (*, paired t-test, $p < 0.05$). There was a significant increase in the rise time in the presence of 1 μm dynorphin in *KOR-cre* positive neurons relative to baseline (*, paired t-test, $p < 0.05$). Data are presented as mean ± SEM.

D. Quantification of the percent change in current amplitude of either *KOR-cre* positive neurons in the presence of dynorphin (1 μm; red), in the presence of norBNI (1 μm; orange), or in the presence of dynorphin (1 μm) and norBNI (1 μm; light orange). n = 3 neurons. Only dynorphin alone caused a significant decrease in the current amplitude (*, paired t-test, $p < 0.05$). Data are presented as mean ± SEM.

2.3.2 KOR is expressed in distinct neurochemical and anatomical subsets of primary afferents

After validating that the *KOR-cre* allele did indeed cause recombination in neurons that express KOR, next we wanted to use neurochemical markers in order to gain a preliminary understanding of the primary afferent subtypes expressing KOR. When visualizing the recombination pattern in *KOR-cre* primary afferent neurons in the DRG, we noted that both large and small diameter cell bodies were labeled (Figure 5E-G). This suggested the possibility that multiple subtypes of primary afferent neurons express KOR. To begin characterizing *KOR-cre* neurons in more detail, molecular markers that are consistently expressed in putative functional groups of primary afferents were used in immunohistochemical (IHC) experiments similar to other reports in the field (McCoy, Taylor-Blake, & Zylka, 2012); (Han et al., 2013); (Bardoni et al., 2014). Calcitonin gene-related peptide (CGRP) was used as a marker for peptidergic neurons

(Basbaum, 2008; Basbaum, Bautista, Scherrer, & Julius, 2009), Neurofilament-200 (NF200) as a marker for myelinated A-δ and A-β fibers (Basbaum, 2008; Basbaum et al., 2009) (L. Li et al., 2011), and tyrosine hydroxylase (TH) which has shown to be expressed by a subset of C-fibers that includes, but is not limited to, C-fiber low-threshold mechanoreceptors (LTMRs) (L. Li et al., 2011; Seal et al., 2009). Quadruple label IHC experiments revealed that the vast majority (~95%) of *KOR-cre* FLEX.ChR2-tdt primary afferents were labeled with at least one of these three markers (Figure 3D). Of these three markers, *KOR-cre*, FLEX.ChR2.tdt neurons co-localized primarily with CGRP-IR (52%) (Figure 3A) or with neurons showing both CGRP-IR and NF200-IR (~12%). *KOR-cre* overlap with NF200-IR neurons was slightly smaller (~23%) (Figure 3B), as was overlap with TH-IR (~8%) (Figure 3E). Little or no overlap was observed of *KOR-cre* neurons with either TRPM8-immunoreactive (IR) or parvalbumin-IR neurons, suggesting that *KOR-cre* does not cause recombination in either cool-sensing neurons (McKemy, Neuhausser, & Julius, 2002; Peier et al., 2002) or in proprioceptors (de Nooij, Doobar, & Jessell, 2013) (Figure 4).

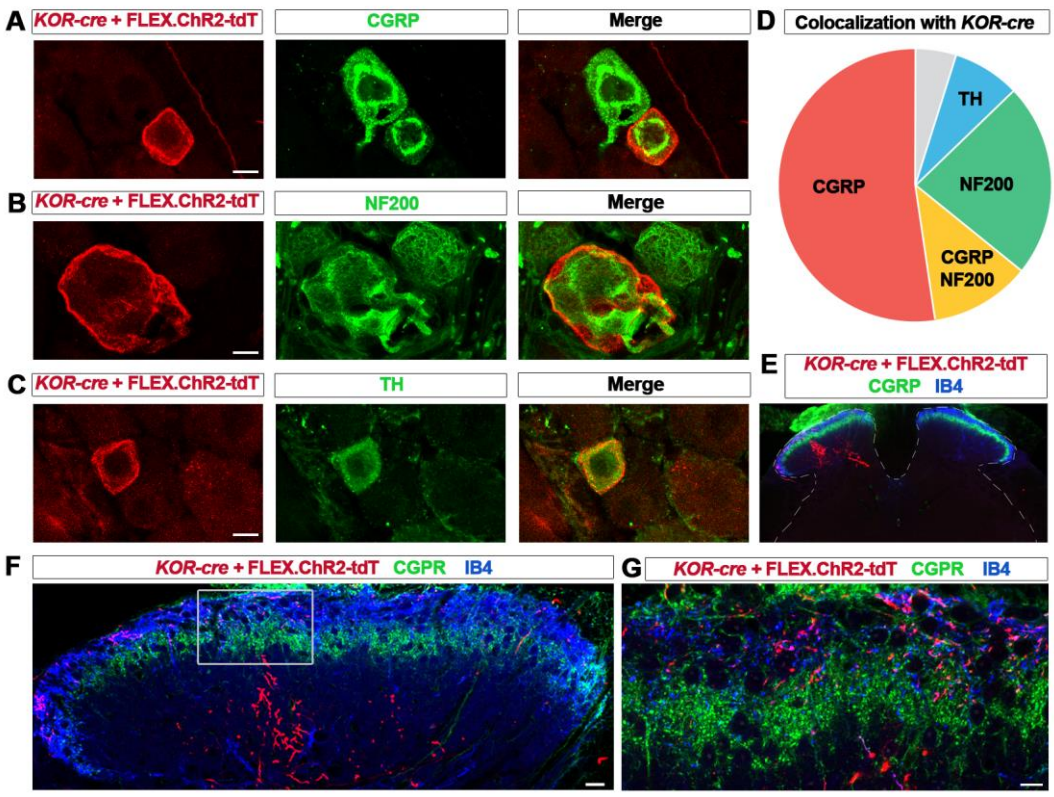


Figure 3. *KOR-cre* labels distinct neurochemical and anatomical subsets of primary afferents

A – C. IHC of lumbar DRG neurons from *KOR-cre* mice labeled using a Cre-dependent virus (AAV.FLEX.ChR2-tdTomato; IT in adult) and co-stained with antibodies to CGRP (A), NF200 (B), and TH (C). Scale bar = 10 µm.

D. Pie chart representing co-localization of immunohistochemical markers with virally-labeled (AAV.FLEX.ChR2-tdTomato, IT in adult) *KOR-cre* DRG neurons. 52% ± 5% of *KOR-cre* neurons co-localize with CGRP (red), 12% ± 6% co-localize with both CGRP and NF200 (yellow), 23 ± 6. % co-localize with NF200 (green), and 8% ± 2% are TH (blue). Only 5% ± 5% of *KOR-cre* neurons did not co-localize with any of these three markers (gray; n = 3 mice).

E – F. Representative images of lumbar spinal cord section of a *KOR-cre* mouse following an injection of AAV.FLEX.ChR2-tdt into the left sciatic nerve at P40. *KOR-cre* + FLEX.ChR2-tdt primary afferent terminals can be seen ipsilateral to the injection in the dorsal horn (E) in both the deeper dorsal horn below the IB4 band (F) and in the superficial dorsal horn where they co-localize with CGRP (G).

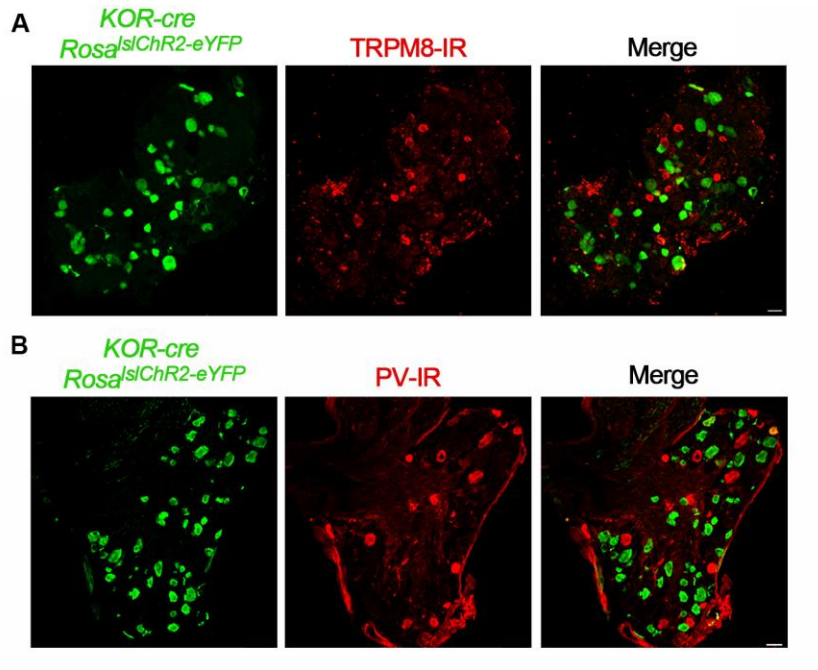


Figure 4. *KOR-cre* labeled afferents to not co-localize with parvalbumin or TRPM8.

A - B. Representative images of lumbar DRG sections showing little to no overlap between *KOR-cre; Rosa*$^{lslChR2\text{-}eYFP}$ positive neurons and TRPM8-IR (A) or parvalbumin (PV-IR; B), indicating that *KOR-cre* neurons do not include putative cool-sensing neurons or putative proprioceptors, respectively.

In a separate triple label IHC experiment, we observed some overlap between *KOR-cre* FLEX.ChR2-tdt neurons and isolectin-B4-IR (IB4) (~11%), a marker expressed largely by non-peptidergic putative nociceptors (Figure 4). However, of those *KOR-cre*, FLEX.ChR2-tdt neurons that were IB4-IR, the overwhelming majority were also CGRP-IR with ~90% of the IB4-IR *KOR-cre* cells also showing IR for CGRP (Figure 5). These results indicate that this group of *KOR-cre* neurons are a specific population of peptidergic IB4-binding cells. These cells, described in Han et al. (Han et al., 2013), show large overlap with a population of primary afferents the authors suggest are specifically tuned for itch sensation.

Many genes show a developmental pattern of expression that is different than that observed in adulthood. As a result, Cre alleles often show altered patterns of reporter expression depending on the developmental timing of recombination. The use of viral recombination provided an opportunity to address whether recombination using the *KOR-cre* allele varies as a function of time in terms of the proportion of neurons in the DRG that show recombination. Further, in combination with the IHC markers described above, it provided an opportunity to address whether *KOR-cre* expression in particular primary afferent subtypes changes across time. Experiments were performed to compare the recombination pattern that is observed when a reporter is introduced at three different stages of development: embryonic (using the Ai9 $Rosa^{lsl\text{-}tdt}$ reporter allele), post-natal day 1 (using AAV viruses delivered IP) (Foust, Poirier, Pacak, Mandel, & Flotte, 2008), and adult (using AAV viruses delivered IT) (Vulchanova et al., 2010); (Dayton et al., 2012).

In general, the distribution of subtypes within the *KOR-cre* population at any of these three times is largely similar. Co-staining with CGRP, NF200 and TH revealed a similar proportion of *KOR-cre* neurons at any time point analyzed showed colocalization with these three markers (Figures 5A and 5B). Co-staining with IB4 and CGRP revealed a larger proportion of IB4-IR only *KOR-cre* neurons labeled in embryonic and early post-natal development, which shifted towards a larger proportion of IB4-IR and CGRP-IR *KOR-cre* neurons in adult labeled neurons (Figure 5A and 5B). This likely indicates that a larger proportion of IB4-IR neurons expressed KOR early in development, but no longer express KOR in adulthood. However, *KOR-cre*- neurons that were labeled when adult mice were infected with Cre-dependent viruses made up a smaller number of the total DRG population than that observed when the recombination occurred during embryonic or early postnatal development (see Figure 5 for further detail). This

analysis revealed that similar proportions of the total *KOR-cre* population co-stained with either CGRP, NF200, or TH across development, but that there was a change from IB4 only *KOR-cre* neurons in early development to IB4 and CGRP *KOR-cre* neurons in adulthood. Thus, while largely similar patterns of recombination are observed, introduction of Cre-dependent reporters into adult *KOR-cre* mice is more specific for neurons that express KOR in adulthood, whereas an embryonic reporter would be useful in an analysis of the developmental expression of KOR.

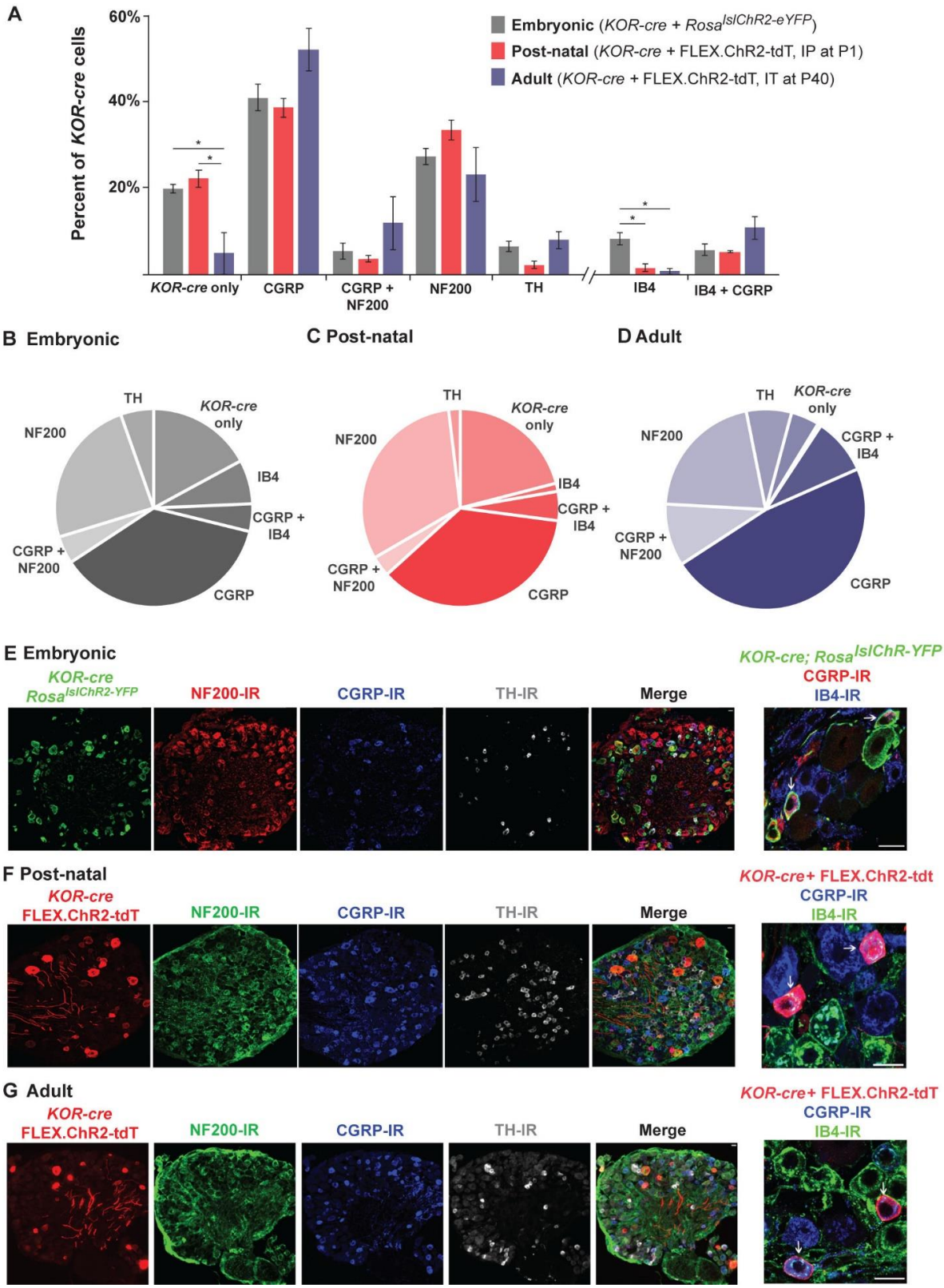


Figure 5. Comparison of *KOR-cre* mediated recombination at different times during development.

A. To determine whether *KOR-cre* mediated recombination varies across an animal lifespan, the recombination that was observed following the introduction of a Cre-dependent reporter at three different stages of development was compared. *KOR-cre* mice were either crossed with Ai9 mice (*Rosa*$^{lslChR2\text{-}eYFP}$), or infected with Cre-dependent virus at either P1 or in adult mice. Sections from DRG were quadruple labeled with the reporter and antibodies against NF200, CGRP, and TH, and the percentage of genetically-labeled cells that co-localize with one or more of these three markers was quantified. Gray bars represent the *KOR-cre* positive population in *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice, red bars represent the *KOR-cre* positive population in mice injected with FLEX.ChR2-tdT virus IP at P1, and blue bars represent the *KOR-cre* positive population in mice injected with FLEX.ChR2-tdT virus IT at P40. When infection occurs in adult, only 5% of labeled *KOR-cre* afferents do not co-localize with either CGRP, NF200 or TH (i.e., *KOR-cre* only). However, if the reporter is introduced during embryonic or early post-natal development, the fraction of *KOR-cre* only afferents is significantly increased (One-way ANOVA with Tukey's multiple comparison's test, *, $p < 0.05$, $n = 3$ mice). In a separate experiment, sections were co-stained with antibodies to CGRP and with IB4. For each condition, *KOR-cre* neurons that co-localized with both CGRP and IB4 were observed. However, when the reporter was introduced embryonically, *KOR-cre* neurons that co-localized with IB4 but not CGRP (i.e., IB4 only) were observed. In contrast, when the reporter was introduced later, a significant decrease in the proportion of *KOR-cre* afferents that co-stain with IB4 only was observed (One-way ANOVA with Tukey's multiple comparison's test, *, $p < 0.05$, $n = 3$ mice). Together, these findings suggest that labeling with the *KOR-cre* allele becomes more restricted over the course of development. Data are presented at mean ± SEM. Similar results were seen whether Cre-dependent viruses were introduced via intrathecal or intrasciatic injection in adult mice ($n = 3$ mice per viral delivery route, data not shown).

B - D. Pie charts representing the proportion of *KOR-cre* neurons that co-localize with various neurochemical markers. Charts illustrate that the proportion of each group remains similar across *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice, *KOR-cre* + FLEX.ChR2-tdT delivered IP at P1 mice, and *KOR-cre* + FLEX.ChR2-tdT delivered IT at P40 mice.

E - G. Representative image of a lumbar DRG section showing co-localization of *KOR-cre* neurons with NF200, CGRP, TH, and IB4 in *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice (E), *KOR-cre* + FLEX.ChR2-tdT delivered IP at P1 mice (F), and *KOR-cre* + FLEX.ChR2-tdT delivered IT at P40 mice (G). Arrows indicate *KOR-cre* positive neurons that co-localize with both CGRP and IB4.

The IHC experiments above suggest that the *KOR-cre* allele mediates recombination in two major populations of primary afferents (CGRP-IR afferents or NF200-IR afferents). Previous work in mice has shown that many CGRP-IR putative nociceptive neurons have central terminals that target the superficial dorsal horn (lamina I) (McCoy et al., 2012) while many myelinated A-δ and A-β fibers, in particular cutaneous myelinated low-threshold mechanoreceptors (LTMRs), have central terminals that target the deeper dorsal horn (lamina III-V) (Abraira et al., 2017). Thus, IHC was used to selectively visualize the central terminals of *KOR-cre*-expressing primary afferents. In order to do so without also labeling *KOR-cre* spinal interneurons, Cre-dependent AAV viruses were either injected into the sciatic nerve of adult mice (Figures 3E, 3F and 3G), or delivered IP at P1 (Figures 6A and 6B). Many *KOR-cre* afferents terminated in the superficial dorsal horn where they co-localized with CGRP. In addition, *KOR-cre* positive afferents terminated in laminae III-IV, the LTMR recipient zone (Abraira et al., 2017). Furthermore, at least some of these fibers appeared to send collaterals in the dorsal funiculus (Figure 6A), consistent with the possibility that *KOR-cre* afferents include LTMRs that target the dorsal column nuclei (Horch, Burgess, & Whitehorn, 1976).

Taken together, the neurochemical and anatomical data support the idea that KOR is expressed by presumed nociceptive peptidergic afferents whose central terminals target lamina I, and presumed LTMRs that target laminae III-IV and the dorsal column nuclei.

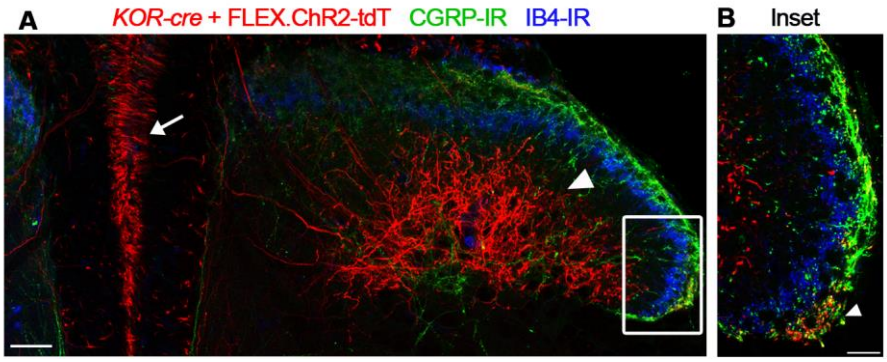


Figure 6. *KOR-cre* labeled afferents include putative LTMRs that target the dorsal columns.

A - B. Representative image of *KOR-cre* primary afferent terminals in the lumbar spinal cord. *KOR-cre* neurons were infected via an IP injection of AAV.FLEX.ChR2-tdT at P1. Arrowhead indicates *KOR-cre* positive terminals in the deeper dorsal horn below the IB4 band (A) and in the superficial dorsal horn that overlap with the CGRP-IR band (B). Arrow indicates *KOR-cre* positive fibers that appear to ascend in the dorsal column pathway. A similar pattern was observed in n = 3 mice.

2.3.3 KOR is expressed in circumferential and lanceolate low-threshold mechanoreceptors

Given there has been no previous indication of a role for KOR signaling in low-threshold mechanosensation, it was somewhat surprising to find *KOR-cre* expression in myelinated, large diameter fibers as well as in *KOR-cre* central terminals in the deeper dorsal horn, consistent with the location of the LTMR recipient zone (Abraira et al., 2017). The possibility of a group of KOR-expressing LTMRs is further supported by the presence of *KOR-cre* fibers in the dorsal column pathway (Figure 6A arrow), as several LTMR subtypes have an axonal branch that ascends along this pathway to the gracile nuclei (Horch et al., 1976).

To investigate the identity of these afferent fibers further, we visualized their peripheral endings using Cre-dependent expression of ChR2-tdTomato in adult mice. In the hairy skin of

the hind paw, we observed that *KOR-cre* labeled afferents formed lanceolate (Figure 7A) or circumferential (Figure 7B) endings around hair follicles. Many of these afferents co-expressed NF200, indicating that they are myelinated, but they did not express CGRP. These results indicate these afferents could be a subset of circumferential endings that respond to light stoking of the skin and not to hair deflection (Bai et al., 2015). In contrast, *KOR-cre* afferents targeting Merkel disks or Meissner corpuscles in glabrous skin were not observed. *KOR-cre* afferents with circumferential or lanceolate endings were also observed in the hairy back skin, where they targeted multiple hair follicle types (Figures 8A and 8B), suggesting that KOR is expressed by a subset of LTMRs that target hair follicles across the body.

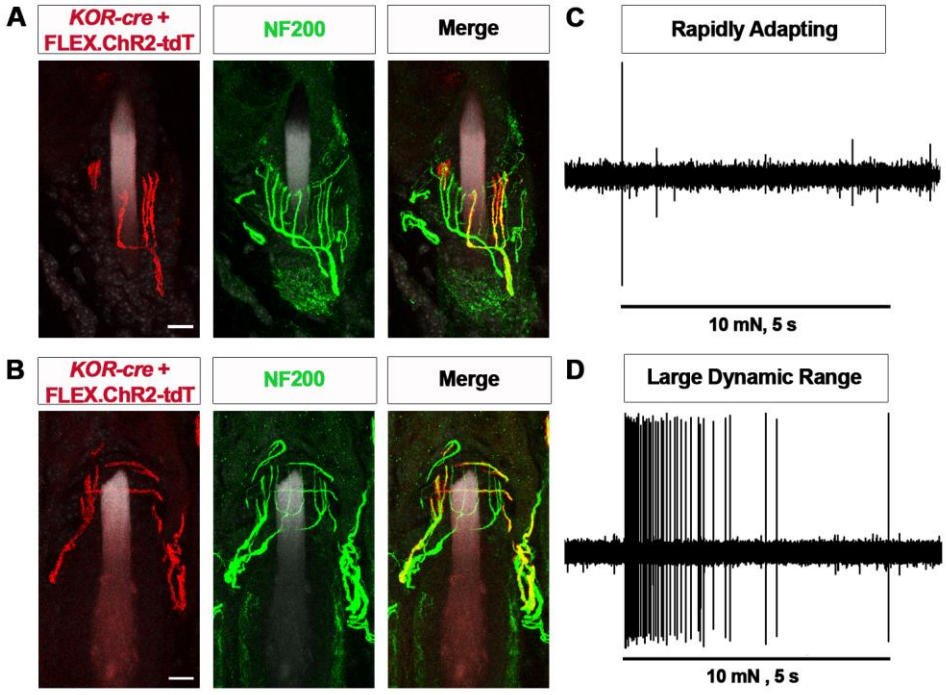


Figure 7. *KOR-cre* mediates recombination of lanceolate afferents and circumferential field receptors

A - B. IHC illustrating examples of a *KOR-cre*-labeled lanceolate fiber (A) or circumferential fiber (B) innervating hair follicles in the hind paw skin (red) and co-localize with NF200 (green). *KOR-cre* afferents were labeled with a Cre-dependent virus (AAV.FLEX.ChR2-tdTomato; IT in adult). Data are representative of n = 2 mice. Scale bar = 10 μm.

C - D. Single fiber recordings were performed in *KOR-cre, ROSA*$^{lslChR2\text{-}eYFP}$ mice and fibers were identified with optogenetic tagging by their response to blue light stimulation of their cutaneous receptive field. A representative trace of a single fiber recordings from a *KOR-cre*-positive rapidly adapting A-fiber (C) and large dynamic range fiber (D) upon application of a mechanical stimulator the receptive field in the skin (5 sec, 10 mN) Data are representative of n = 4 cells of each type.

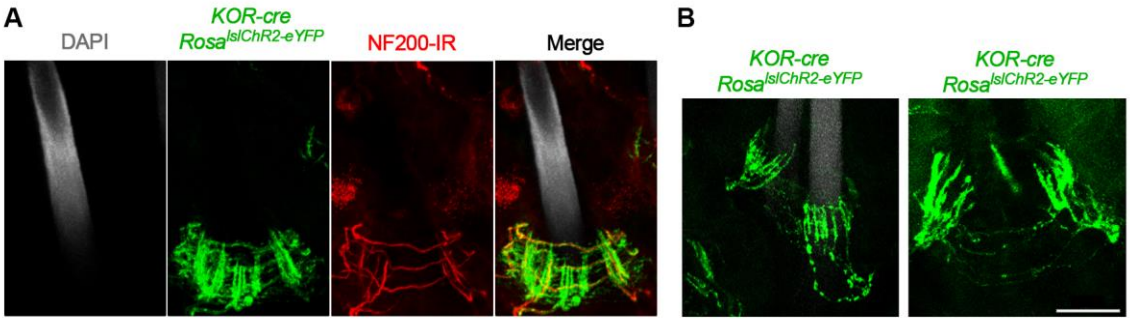
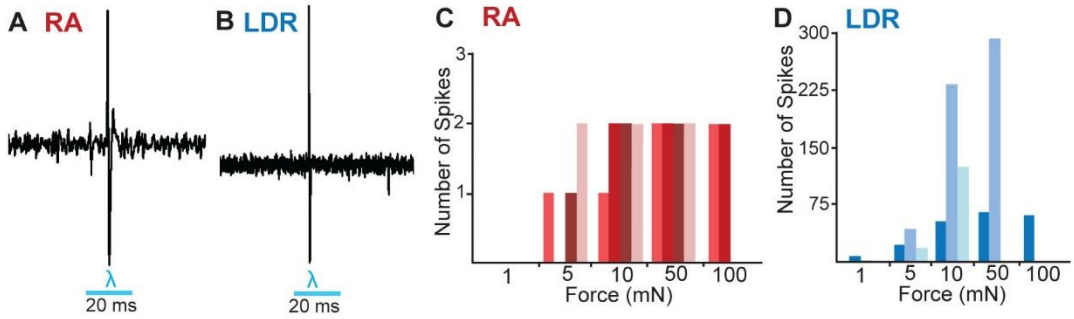


Figure 8. *KOR-cre* labeled afferents form circumferential and lanceolate endings that target multiple hair types

A - B. Wholemount IHC of the thoracic skin from a *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mouse co-stained showing *KOR-cre* positive lanceolate (left), and lanceolate and circumferential endings (right) innervating hair follicles in the back skin of a guard hair (A) and zigzag and/or awl/auchene hairs (B).

To characterize the physiology of low-threshold KOR-cre afferents, we collaborated with the Koerber laboratory to use the ex vivo skin-nerve preparation and teased fiber recordings (McIlwrath et al., 2007). ChR2-expressing *KOR-cre* afferents were identified through their response to optogenetic stimulation (Figures 9A and 9B), and then characterized by their responses to the application of a range of constant mechanical forces (1 – 100 mN). Using this approach, afferents with two firing characteristics were observed. The first responded to low-threshold stimulation (e.g., 5 or 10 mN), and consistently fired only once (at stimulus onset) or twice (at stimulus onset and offset) in response to mechanical stimulation throughout the range

of forces tested (Figures 7C, 9C and 9E). This electrophysiological signature is consistent with the possibility that *KOR-cre* afferents include rapidly adapting lanceolate fibers (Koerber & Woodbury, 2002); (L. Li et al., 2011). The second group also responded to low-threshold stimulation (Figures 7D, 9D and 9E). However, these afferents fired throughout the period of mechanical stimulation. Moreover, they showed dramatically increased firing rates in response to increasing forces, firing as many as 300 action potentials over 5 s (Figures 9E). These characteristics (low-threshold but responsive across a large dynamic range) are consistent with the idea that these fibers are afferents that form circumferential endings around hair follicles (Bai et al., 2015). Thus, these observations support the idea that KOR is expressed by two sensory neuron subtypes that target hair follicles.



E

			Number of Spikes					
Type	CV	Threshold	1mN	5mN	10mN	25mN	50mN	100mN
RA	5.63	5	0	1	1	2	2	
RA	13.89	10		0	2	2	2	
RA	6.64	5	0	1	2	2		
RA	4.61	5	0	2	2	2		
LDR	13.28	5	0	7	22	53	65	63
LDR	6.67	5	0	1	43	233	294	
LDR	8.81	5		1	*	*	*	*
LDR	7.03	10		0	18	125		

*There is a second cell present in the recording and making accurate spike counting difficult.

Figure 9. Electrophysiological characteristics of myelinated *KOR-cre* afferents

Teased-fiber recordings were performed in a skin-nerve preparation of the dorsal hindpaw from *KOR-cre; Rosa*$^{lslChR2\text{-}eYFP}$ *mice.*

A - B. Optogenetic tagging was performed to identify *KOR-cre* afferents. Example traces of responses to blue light stimulation (470 nm, 20 ms) of the receptive field of afferents that were subsequently characterized as rapidly adapting (RA, A) or as large dynamic range (LDR; B).

C - D. Graph of the number of spikes observed following mechanical stimulation of the receptive field with a piezo-electric stimulator at varying forces. Each shade represents an individual cell's response across stimulus intensities. Rapidly adapting afferents fired only 1 or 2 spikes, irrespective of the force (C); large dynamic range fibers showed an increase in the spike number as a function of force.

E. Table displaying the measured conduction velocity (CV), mechanical threshold, and the number of spikes following a given stimulus for each *KOR-cre* neuron recorded.

2.3.4 KOR is expressed in a transcriptionally distinct subset of peptidergic afferents that target multiple tissue types

The majority of sensory neurons targeted by the *KOR-cre* allele were not LTMRs, but rather peptidergic afferents, which are known to target cutaneous tissue but other peripheral tissues as well. As previous work from our laboratory and others have shown involvement of the kappa opioid system in regulating pruritoception (in addition to a role in nociceptive processing), and itch sensation arises in the skin, the skin from *KOR-cre* $Rosa^{lsl\text{-}tdt}$ mice was co-stained with PGP9.5 (a marker for primary afferent neurons). *KOR-cre* afferents co-localized with PGP9.5 and formed free nerve endings (Figure 12A) that traveled through the dermis into the epidermal layer of both the hairy and glabrous hind paw skin (Figure 11). However, they were not specific to the skin, since we observed labeled afferents in every nerve examined, including saphenous nerve, femoral nerve, sciatic nerve, L6 nerve, and the infraorbital nerve (Figures 10A - 10E). Further, *KOR-cre* FLEX.ChR2-tdt (IT injection) positive fibers were present in both the

saphenous and femoral nerves which innervate selectively cutaneous and muscular tissue of the hindlimb, respectively, indicating that it is highly likely primary afferents expressing KOR in adulthood target at least the skin and muscle (Figure 10F - 10G). Free nerve endings were also observed in visceral tissue, such as the lamina propria and muscularis layers of the bladder wall (Figure 10H). These findings indicate that KOR-expressing afferents likely innervate multiple tissue types throughout the body, and are therefore not limited to a specific peripheral target and could be involved in detecting nociceptive and pruritoceptive information at the level of the periphery.

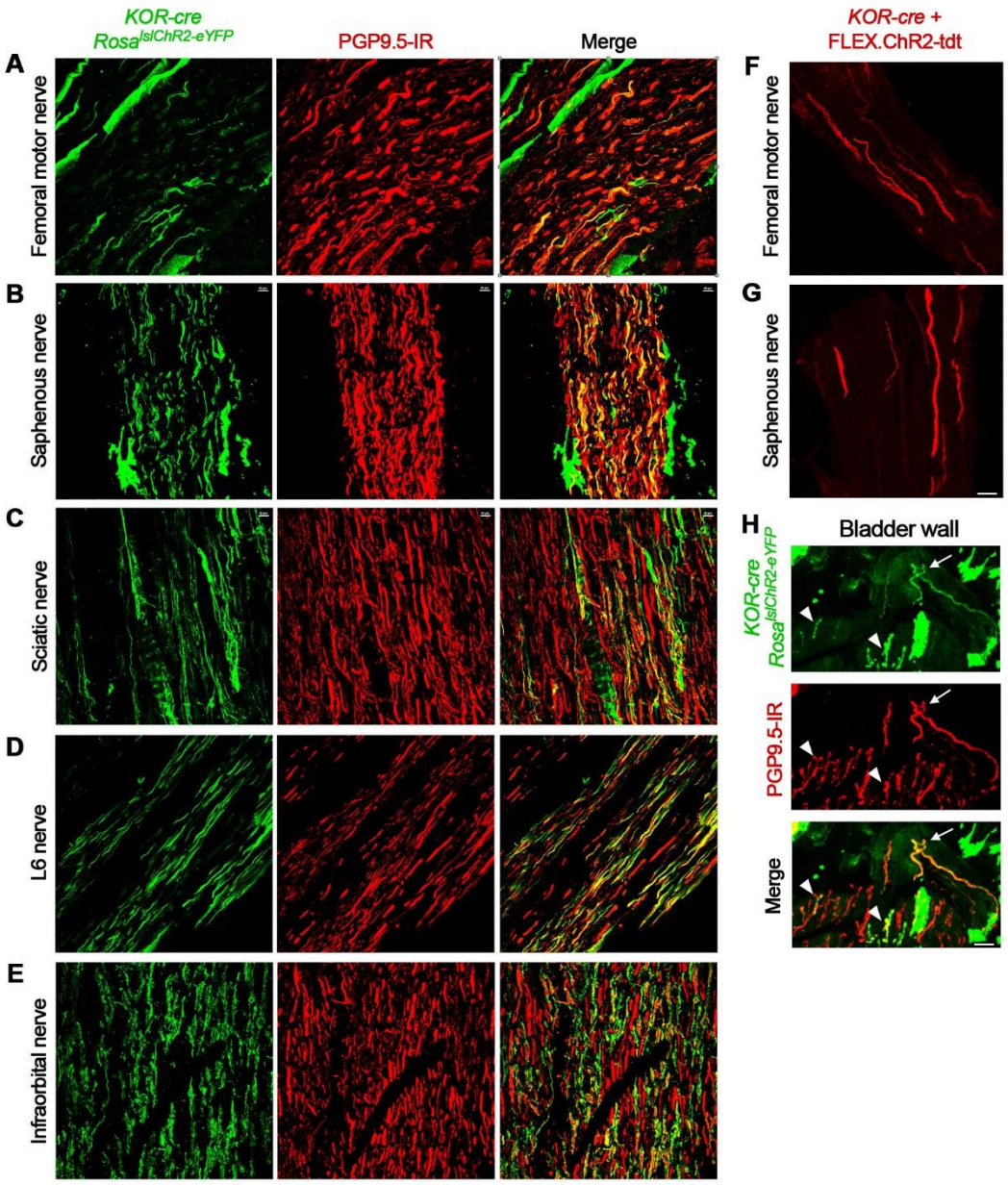


Figure 10. *KOR-cre* afferents target tissues throughout the body

A – E. Representative images of IHC from *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice showing *KOR-cre* positive fibers that co-localize with PGP9.5-IR in the femoral motor nerve, (A) saphenous nerve (B), sciatic nerve (C), L6 nerve (D) and the infraorbital nerve (E). These data suggest that KOR-expressing neurons innervate multiple tissue types throughout the body.

F – G. Representative images of IHC from *KOR-cre* + FLEX.ChR2-tdt delivered IT at P40 mice in the femoral motor nerve (F) and the saphenous nerve (G) that is consistent with the pattern observed in *KOR-cre; Rosa*$^{lslChR2\text{-}eYFP}$ mice.

H. Representative images of IHC from *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice showing *KOR-cre* positive fibers that co-localize with PGP9.5-IR in the bladder wall that terminate in both the lamina propria (arrows) and the muscularis (arrowheads).

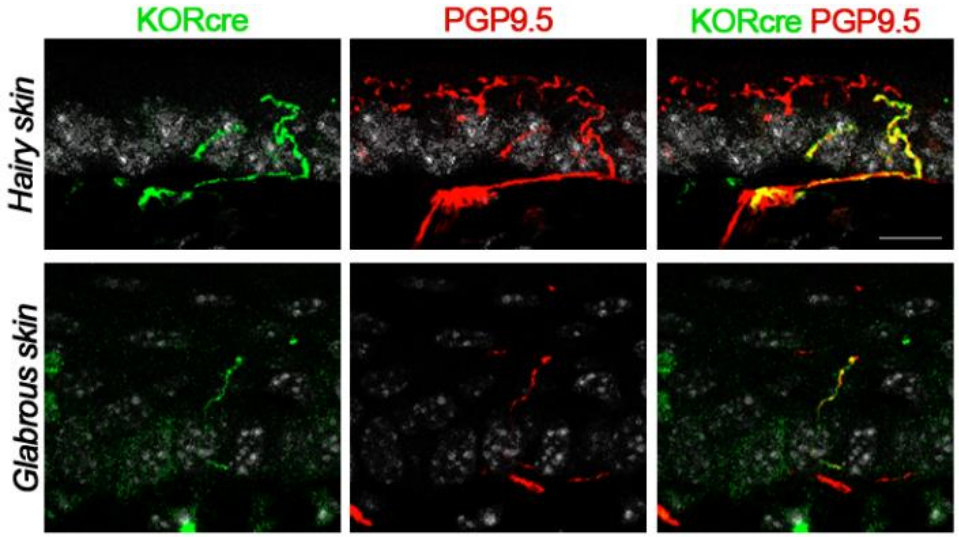


Figure 11. *KOR-cre* afferents form free nerve endings in the hairy and glabrous skin of the hind paw

Representative images of IHC from *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice showing *KOR-cre* positive fibers that co-localize with PGP9.5-IR in the hairy skin of the hind paw (top) and the glabrous skin of the hind paw (bottom). Scale bar = 10μm.

Consistent with previous literature that strongly suggests a role for KOR in peripheral modulation of nociception (for review, see (Vadivelu, Mitra, & Hines, 2011); (Vanderah, 2010)), *KOR-cre* expression is observed in putative peptidergic nociceptive (CGRP-IR) primary afferents (Figure 12B and 12C). Additionally, a high overlap with *KOR-cre*, FLEX.ChR2-tdt expression with other markers of putative peptidergic nociceptors was noted, such as substance P (~51%) (Figure 12B and 12C) and cation channel TRPV1 (~53%) (Figure 12B and 12C). While

many *KOR-cre* neurons expressed these classic 'peptidergic' markers many (Figure 12B arrows), but not all (Figure 12B arrowheads), of the peptidergic neurons co-localized with *KOR-cre*. Even when using a Cre-dependent tdTomato allele as a reporter to mark all cells that have ever expressed KOR at any time during development, only half of CGRP-expressing afferents were labeled (Figure 5E). This observation raises the question of whether or not the peptidergic neurons expressing KOR are a unique subset of this peptidergic population.

To investigate the features that distinguished peptidergic *Oprk1*-positive neurons from peptidergic *Oprk1*-negative neurons, we turned to single cell quantitative RT-PCR. To enable characterization of CGRP-expressing subtypes, analysis was restricted to afferents that expressed *Calcα* (which encodes CGRP). First, we compared 15 *KOR-cre* positive afferents, in which the presence of *Oprk1* message was subsequently confirmed, to 20 *KOR-cre* negative neurons with single cell RT-PCR (Figure 12D). This analysis revealed a number of peptidergic markers that were expressed at significantly higher levels in peptidergic afferents that express *Oprk1* relative to peptidergic afferents that do not (Figures 12E – 12J). *Oprk1*-expressing afferents showed ~12-fold higher *Calcα* and ~6-fold higher *Tac1* expression on average, genes that encode for the neuropeptides CGRP and Substance P. *Oprk1*-expressing afferents also showed increased expression of transcripts encoding for several receptors; an ~4-fold increase in *Trpv1*, ~18-fold increase in *Gfrα3*, ~3-fold increase in *TrkA*, and ~3-fold increase in *Ptgir*. These findings suggest that, within the peptidergic class of primary afferents, KOR is preferentially expressed in a transcriptionally distinct subset of neurons that express high levels of markers for classically peptidergic afferents.

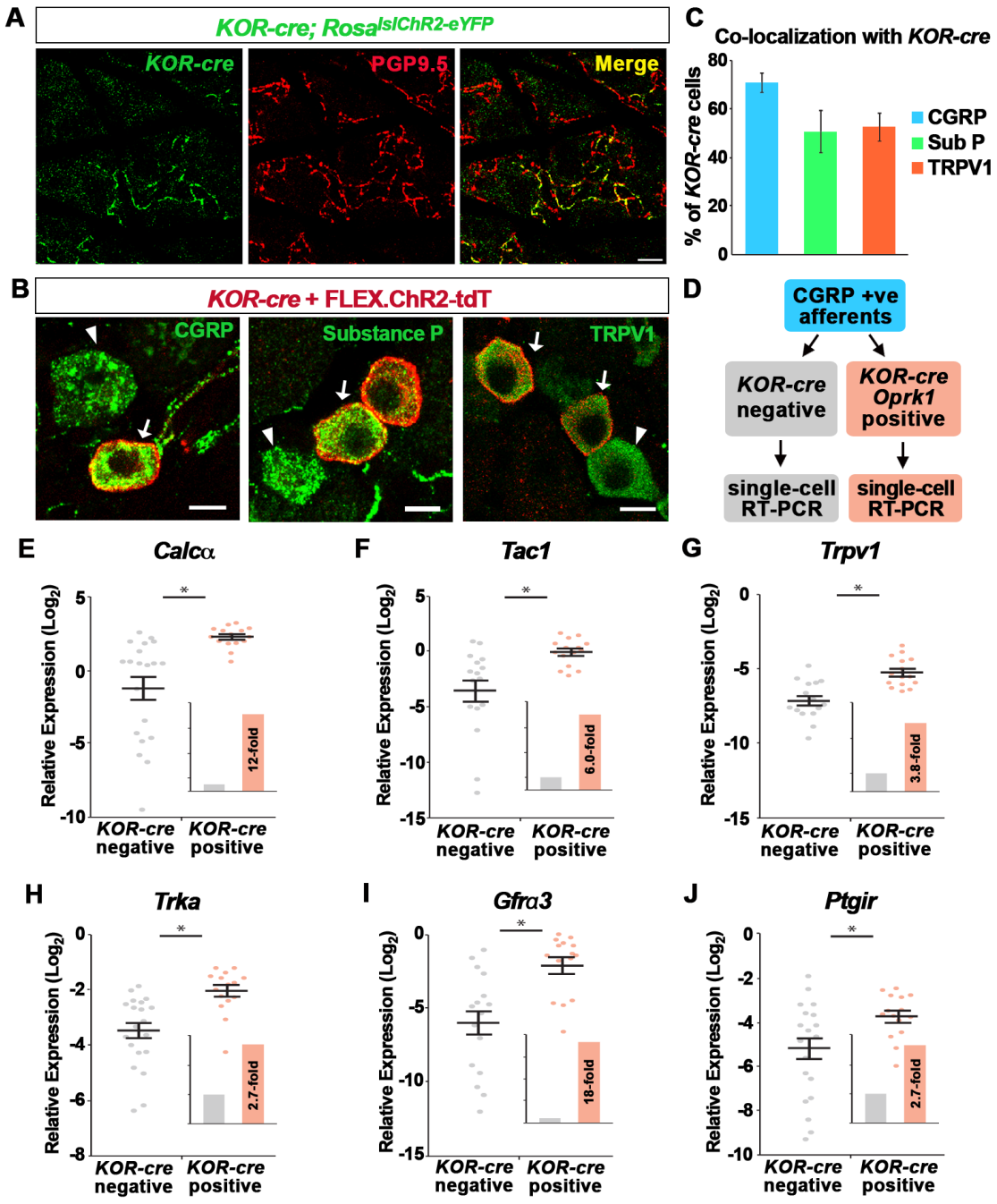


Figure 12. KOR is expressed by a transcriptionally distinct subset of peptidergic DRG neurons

A. Wholemount IHC of the thoracic skin from a *KOR-cre; Rosa*$^{lslChR2\text{-}eYFP}$ mouse showing that *KOR-cre*-positive afferents (green) make up a subset of free nerve endings that terminate in the epidermis, as assessed by co-localization with PGP9.5 (red). Scale bar = 10 µm.

B. IHC of DRG showing *KOR-cre*-labeled neurons co-localize with markers of peptidergic neurons. *KOR-cre* afferents were labeled with a Cre-dependent virus (AAV.FLEX.ChR2-tdTomato; IT in adult) Arrows indicate co-localization; arrowheads indicate CGRP-, Substance P-, or TRPV1-expressing neurons that are not *KOR-cre* positive. Scale bar = 10 μm.

C. Quantification of (B) showing the percentage of *KOR-cre* + FLEX.ChR2-tdT cells that co-localize with peptidergic markers. Data are presented as mean ± SEM (n = 3 mice).

D. Schematic of single-cell RT-PCR experimental design. Lumbar DRG neurons from *KOR-cre; Rosa*$^{lslChR2\text{-}eYFP}$ mice were collected individually. Only peptidergic cells that expressed *Calcα* were analyzed. Transcript levels were compared between *KOR-cre*-labeled neurons that showed clear *Oprk1* expression and *KOR-cre* negative neurons.

E - J. Expression levels of *Calc* (E), *Tac1* (F), *Trpv1* (G) *TrkA*, (H), *Gfr 3* (I) and *Ptgir* (J) mRNA relative to *GAPDH* in *KOR-cre* negative (grey) and *KOR-cre* positive (orange) DRG neurons. Data are presented as the $-\log_2$ ΔCT expression relative to *GAPDH* expression within the same cell such that bigger numbers represent higher mRNA expression. There was a significantly higher relative expression level of each transcript in *KOR-cre* positive neurons compared to *KOR-cre* negative neurons (Student's t-test, $p < 0.05$). Inset shows fold-increase in the average expression level normalized to the average expression in *KOR-cre* negative neurons. Black bars represent mean ± SEM and colored dots represent data pionts from individual cells (n = 20 *KOR-cre* negative neurons, n = 15 *KOR-cre* positive neurons).

The transcriptional profile of KOR afferents could provide additional information for a potential functional role for these neurons. However, this would be further informed by understanding the peripheral innervation target and how that relates to the transcriptional profile. While the experiments above show that *KOR-cre* fibers innervate multiple tissue types, and that some transcripts are highly expressed in KOR-expressing afferents, it is not known whether the transcriptional profile is similar across KOR-expressing afferents that target different tissues. This information could provide insight into whether KOR-expressing afferents play a similar role in somatosensory processing across the body, and would provide insight into the effects of KOR

agonist on modulating somatosensation in different pathological conditions. In order investigate this further, the transcriptional profile of *Rosa*$^{lsl\text{-}tdt}$ positive or *Rosa*$^{lsl\text{-}tdt}$ negative neurons from *KOR-cre mice* backlabeled from either the saphenous or femoral nerve were analyzed. Fluorescent WGA was used as a backlabeling agent as it is preferentially transported by peptidergic afferents and therefore increased the likelihood of labeling this type of afferent for the analysis. When comparing all afferents backlabeled from the saphenous nerve to all afferents backlabeled from the femoral nerve (regardless of *KOR-cre* expression), there was generally higher expression of *Calcα* and *Ptgir* in femoral cells than in saphenous cells (Figure 13), consistent with the concept that afferent targeting different tissue types are unique in their properties. Consistent with the previous experiment (Figure 12 E-J), there was a greater proportion of *KOR-cre Rosa*$^{lsl\text{-}tdt}$ positive neurons expressing high levels of *Calcα*, *Tac1*, *Trpv1*, *Trka*, *Gfrα3*, and *Ptgir* (Figure 13) compared to *KOR-cre Rosa*$^{lsl\text{-}tdt}$ negative neurons backlabeled from either the saphenous nerve or femoral nerve (Figure 13). This qualitative assessment supports the conclusion that *KOR-cre* positive neurons express higher levels of particular transcripts regardless of whether they target the skin or the muscle.

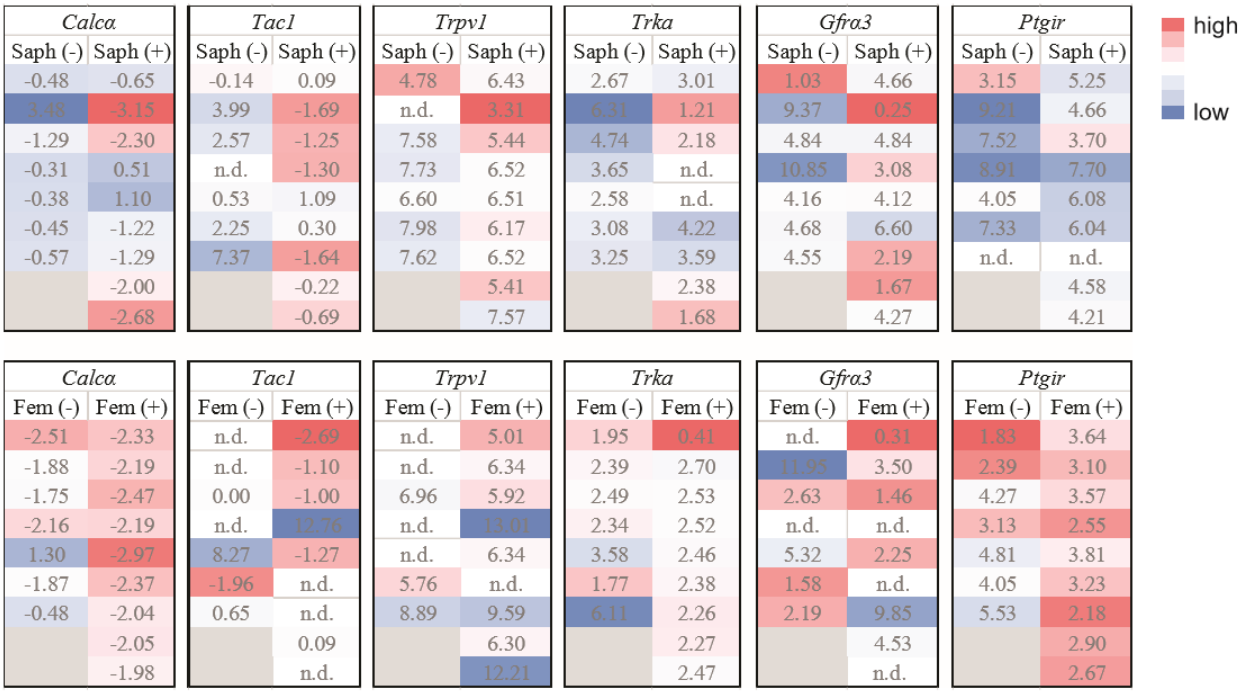


Figure 13. *KOR-cre* afferents targeting muscular or cutaneous tissue express similar transcriptional profiles

Table comparing transcript levels of *KOR-cre Rosa*^{lsltdt} negative (-) or *KOR-cre Rosa*^{lsltdt} positive (+) afferents backlabeled from either the saphenous (Saph) or femoral (Fem) nerves using WGA-488. Afferents were included in the table based on expression of *Calcα* in order to compare the *KOR-cre Rosa*^{lsltdt} negative (-) or *KOR-cre Rosa*^{lsltdt} positive (+) afferents within the peptidergic afferent population. Each cell in the table represents the ΔCT (Log_2 Transcript/GAPDH) for the given transcript in an individual afferent. The heat map is representative of relative expression within a transcript column across tissue target such that the lowest ΔCT value for *Calcα* (regardless of backlabel) is blue and the highest is red.

Saph (-) = Saphenous backlabeled, *KOR-cre Rosa*^{lsltdt} negative; n= 7;

Saph (+) = Saphenous backlabeled, *KOR-cre Rosa*^{lsltdt} postitive; n=9;

Fem (-) = Femoral backlabeled, *KOR-cre Rosa*^{lsltdt} negative; n= 7;

Fem (+) = Femoral backlabeled, *KOR-cre Rosa*^{lsltdt} postitive; n=9;

n.d. = not detected.

2.4 DISCUSSION

In this chapter, we have provided evidence that supports the use of *KOR-cre* allele to identify, target, and characterize primary afferents that express KOR in adult mice. When used in combination with a cre-dependent virus that leads to fluorescent reporter expression in adult *KOR-cre* primary afferents, experiments showed that the majority of the cells labeled using this approach express *Oprk1* mRNA, and those that are not labeled do not express *Oprk1*, via single-cell RT-PCR or dual FISH. Whole-cell patch clamp recordings from acutely dissociated DRG neurons demonstrated that *KOR-cre* neurons also express functional KOR, as dynorphin decreased the magnitude of VGCC in many *KOR-cre* neurons, but not in *KOR-cre* negative neurons. These experiments suggest that *KOR-cre* primary afferents are highly likely to express KOR, and that this approach does not exclude a large population of KOR-expressing neurons. Therefore, the *KOR-cre* allele was used as a tool to identify and characterize KOR-expressing primary afferent neurons. Through a combination of immunohistochemistry and single cell RT-PCR, at least two distinct subtypes of KOR-expressing primary afferent neurons were identified. One is a group of transcriptionally distinct peptidergic afferents that centrally target the superficial dorsal horn and peripherally target multiple tissues including skin, muscle, and viscera. The other is a group of LTMRs that centrally target the deeper dorsal horn and dorsal column, and peripherally form circumferential and lanceolate endings around hair follicles in the skin.

One of the most surprising findings was KOR expression in a subgroup of LTMRs. To the best of our knowledge, KOR expression in LTMRs has not been described previously. The past work investigating KOR mRNA or protein expression in the DRG has described the localization to primarily be in small-to-medium diameter neurons. As most LTMRs have

medium-to-large diameter somas (Le Pichon & Chesler, 2014), this finding was unexpected. The previous data aren't necessarily inconsistent with the results from the experiments above, though, as several reports state that most of the KOR primary afferents were small diameter, but not all of the afferents were small diameter (Ji et al., 1995); (M. K. Schafer et al., 1994); (Mansour et al., 1996). Further, the large-diameter somata in the DRG that were *KOR-cre* positive were relatively low in number. The total number of *KOR-cre* neurons labeled in adulthood were a qualitatively low percentage of the total number of neurons (personal observation) which is consistent with a previous report from Ji et al. (Ji et al., 1995) that reports approximately 9% of DRG neurons expressed KOR-IR. Hence a small proportion of an already small group of afferents is not likely to warrant abundant description.

While previous descriptions of KOR on LTMRs is lacking, work from the laboratory of Gregory Scherrer has suggested that the DOR is expressed by a subgroup of LTMRs, namely NF200-IR circumferential fibers in the hairy skin and fibers innervating Meissner's corpuscles in the glabrous skin (Bardoni et al., 2014). However, the report from this group is controversial as they use a DOR-eGFP knockin mouse and there is debate as to whether addition of the eGFP sequence to the DOR protein alters expression pattern in terms of localization both within the cell and across cell types (H. B. Wang, Guan, Bao, & Zhang, 2008). As the main experiment identifying LTMRs as expressing DOR used this mouse line, further evidence may be needed to definitively determine if DORs modulate low-threshold mechanosensation. The studies investigating the expression of opioid receptors on LTMRs have drawn attention to the possibility that the opioid receptor system may play a more nuanced role in modulating somatosensation than the previous focus on the regulation of pain and itch. Interestingly, a few patients in clinical trials with the peripheral KOR agonist CR845 report paresthesia as a side

effect of the drug (personal communication). Although there are many possible underlying causes, it is tempting to speculate that this may be a result of the activation of KORs that are expressed by LTMRs.

In addition to a subtype of LTMRs, KOR-expressing afferents also included a subset of peptidergic neurons that likely play a role in the transmission of pruritoceptive information from the skin and nociceptive information from tissues across the body. Gebhart and colleagues have previously investigated the effects of different classes of opioid receptor agonists on the primary afferents innervating the colon or the bladder and reported that a KOR agonist, but not MOR or DOR agonists, decreased responses of afferent fibers to distention (Sengupta, Su, & Gebhart, 1996); (Su, Sengupta, & Gebhart, 1997a, 1997b). This suggested the possibility of differential tissue-type distribution of opioid receptor classes; KOR could be more highly expressed in the viscera than the other opioid receptor classes. Consistent with this hypothesis is the observed efficacy of KOR agonists in reducing behavioral responses in pre-clinical models of visceral pain (for review, see (Riviere, 2004); (Davis, 2012)) and in clinical trials in patients with irritable bowel syndrome (Mangel & Hicks, 2012); (Fant et al., 2017). However, the Gebhart lab discovered that the effects they observed in visceral afferents were due, at least in part, to arylacetamide KOR agonists' effects on voltage-gated sodium channels (Su et al., 2009; Su, Joshi, Kardos, & Gebhart, 2002); (Joshi, Lamb, Bielefeldt, & Gebhart, 2003). This doesn't necessarily rule out KOR expression in visceral afferents, but it does call into question a possible target tissue specificity. In the experiments described above, we observed *KOR-cre* labeled primary afferent nerve fibers in every nerve examined, supporting the idea that KOR-expressing fibers target multiple tissue types including cutaneous, muscular and visceral tissues. Quantifying the innervation density of KOR-expressing fibers in each type of target tissue would

be an interesting way to explore any possible target tissue preference for KOR-expressing afferents, and could provide further clues as to the role KORs play in modulating somatosensory information from across the body. Yet this type of experiment is difficult to perform given the current tools available; the *KOR-cre Rosa*^{lsltdt} mouse line labels some afferents that no longer express the KOR in adulthood, and using the viral approach to label *KOR-cre* expressing afferents in the adult requires either an intrathecal or intraneural injection which is never 100% efficient. Thus, both approaches are not suited to answer this type of question.

Although we were not able to use the *KOR-cre* allele to quantify the innervation density in different target tissues, we did find that both saphenous and femoral backlabeled *KOR*-cre afferents expressed relatively high levels of a particular set of transcripts when compared to other peptidergic afferents. *Oprk1*-expressing peptidergic afferents expressed significantly higher levels of the transcripts *Calcα*, *Tac1*, *Trpv1*, *Trka*, *Gfrα3*, and *Ptgir* than the non-*Oprk1* peptidergic neurons, indicating that KOR-expressing afferents are a distinct subset of peptidergic afferents. Interestingly, several of these transcripts encode proteins that have been previously shown to be involved in mechanical and / or thermal hyperalgesia. Intraplantar injection of nerve growth factor (NGF), a ligand of TrkA, causes an increase in mechanical and thermal sensitivity, which can be partially blocked by a TRPV1 antagonist (Mills et al., 2013). A study from Moriyama et al. (Moriyama et al., 2005) suggests that PGI_2 receptors (encoded by *Ptgir*) on primary afferents contribute to nociception, as PGI_2 pretreatment has been shown to potentiate capsaicin-evoked inward currents and decrease the temperature threshold for TRPV1 activation in dissociated mouse DRG neurons (Moriyama et al., 2005). Consistently, this study showed that an intraplantar injection of PGI_2 caused thermal hyperalgesia in the Hargreaves' test. Further, prostacyclin administration in cultured rat primary afferents amplified release of CGRP and SP

(Hingtgen & Vasko, 1994). Many studies have shown that the peptides CGRP and SP play a large role in neurogenic inflammation. Neurogenic inflammation is inflammation resulting from activation of peripheral nerve fibers which causes release of the neuropeptides CGRP and SP, which subsequently cause plasma leakage and vasodiliation (Baluk, 1997). This type of inflammation induces hypersensitivity in addition to swelling and redness (for review, see (Richardson & Vasko, 2002)). Therefore, it is likely that most KOR-expressing peptidergic afferents are a subset of nociceptors activated by a particular set of chemical mediators and likely to be sensitized (or play a role in the sensitization of other afferents) following an injury or in an inflammatory state.

In addition to the possible role in nociceptive processing and sensitization, *KOR-cre* afferents also showed IR that suggests KOR could be expressed by at least some primary afferents involved in pruritoception, which would be consistent with previous literature showing that peripherally-restricted KOR agonists inhibit scratching (Inan & Cowan, 2004); (Cowan et al., 2015). In terms of IHC staining, CGRP and IB4 are commonly used markers to distinguish between 'peptidergic' and 'non-peptidergic' afferents. When speaking in broad generalizations, peptidergic neurons are thought to be involved in the transmission of noxious thermal and chemical information, while non-peptidergic neurons are thought to be involved in the transmission of noxious mechanical information. These two groups are therefore generally referred to as non-overlapping, functionally distinct populations. While most CGRP-IR and IB4-IR do not colocalize, a report by Han et al. (Han et al., 2013) described a small population of primary afferents that show colocalization with both markers. This study was characterizing primary afferents that express the Mas-related G-protein receptor A3 (MrgprA3), and approximately 65% of MrgprA3-cre neurons colocalized with both CGRP and IB4. Interestingly,

a ligand for this receptor is the anti-malarial drug and widely-used pruritogen, chloroquine. This group conducted further experiments whose results suggest that MrgprA3-expressing neurons are specifically tuned to transmit pruritoceptive information. As the small group of IB4-IR KOR-cre neurons in adulthood were also predominantly CGRP-IR, it is interesting to consider the possibility that some of the MrgprA3 afferents may express KOR which would be consistent with the idea that kappa opioids modulate itch sensation at the periphery as well as at the level of the spinal cord (Kardon et al., 2014). However, *MrgprA3* mRNA was not highly detected in *Oprk1* expressing afferents in the single-cell RT-PCR experiments. Due to the low number of *Oprk1* expressing primary afferent neurons that were analyzed, though, it is difficult to draw any definitive conclusions. The evidence suggests the possibility that KOR is expressed on a subset of MrgprA3 afferents, but more experiments would be needed to test this prediction. Further, MrgprA3 afferents are only one subgroup of primary afferent fibers that have been proposed to be involved in detecting pruritoceptive stimuli (Mishra & Hoon, 2013); (Sun et al., 2009); (Usoskin et al., 2015); (Azimi et al., 2017) and future experiments could further delineate the expression of KOR on various itch subgroups and the role peripheral KOR agonists play in modulating itch sensation that arises from specific pruritogens.

3.0 THE EFFECT OF KOR AGONISTS CENTRALLY AND PERIPHERALLY

The work described within this chapter is part of a manuscript that is under review at the journal *Neuron*. The draft of manuscript was prepared by myself and Dr. Sarah Ross, with review and editing by Drs. Rick Koerber and Michael Gold. Junichi Hachisuka, Yu Omori and myself designed, conducted, and analyzed the data from the recordings of spinal neurons in lamina I. Yu Omori developed the modified whole spinal cord preparation, conducted and analyzed the data from the recordings of spinal neurons in lamina III. All behavioral experiments were conducted by myself and Stephanie Fulton, with the exception of the Hargreaves' test in *KOR mutant* and wild-type mice which was conducted by Huizhen Huang. Figures were prepared by myself and edited by Dr. Sarah Ross.

3.1 INTRODUCTION

Primary sensory afferents are pseudo-unipolar neurons with a single axon leaving the cell body, which then bifurcates; sending one branch to the dorsal horn of the spinal cord and the other branch to innervate a target tissue in the periphery. Previous studies have shown evidence that KORs in primary afferents are expressed on central and peripheral terminals and affect somatosensory transmission at either or both locations (Ji et al., 1995); (Labuz et al., 2009); (Stevens & Seybold, 1995); (Besse, Lombard, Zajac, Roques, & Besson, 1990a, 1990b).

Understanding the types of primary afferents that express KOR provides an opportunity to investigate the effect of KOR signaling on somatosensory transmission with more granularity.

Several studies performed previously in the substantia gelatinosa of the rat spinal cord have investigated whether presynaptic KORs on central primary afferent terminals could decrease neurotransmitter release onto spinal cord neurons (Ikoma, Kohno, & Baba, 2007); (Kohno et al., 1999); (Randic et al., 1995); (Ueda et al., 1995). However, the results of these studies provide evidence that is both consistent and inconsistent with this possibility. Randic et al. (Randic et al., 1995) found that most of the modulation of dorsal horn neurons was due to KOR agonists' action on post-synaptic receptors, but did observe evidence for presynaptic modulation in a smaller population of dorsal horn neurons. Experiments performed by Kohno et al. (Kohno et al., 1999) and Ikoma et al. (Ikoma et al., 2007) detected presynaptic inhibition of Aδ and C fiber-evoked EPSCs using either MOR or DOR agonists, but not a KOR agonist. Ueda et al. (1995) showed similar results, such that MOR and DOR agonists reduced capsaicin-induced glutamate release in the dorsal horn, but KOR agonists did not. Further, several studies measuring neuropeptide release report no effect of KOR agonists on either SP (Zachariou (Zachariou & Goldstein, 1996); (Hirota et al., 1985) or CGRP (Collin et al., 1993); (Ballet et al., 1998) levels in the dorsal horn following primary afferent stimulation.

Anatomical and histological studies, however, support the presence of KORs on pre-synaptic primary afferent terminals in the dorsal horn (Harris, Chang, & Drake, 2004); (Stevens & Seybold, 1995); (Ninkovic, Hunt, & Kelly, 1981); (Besse et al., 1990a, 1990b), so it is surprising that several studies report no effect of KOR agonists on neurotransmitter or neuropeptide release from primary afferents into the dorsal horn. Two methodological reasons could underlie this seemingly contradictory evidence. First is that a small proportion of primary

afferents express KOR. The studies discussed above primarily used methods that would activate a large number of primary afferents, such as electrical stimulation or capsaicin application. Second is that many of these studies were measuring neurotransmitter release in the substantia gelatinosa of the rat. Granted there are anatomical differences between the rat and the mouse, but the results in previous chapter show the most dense *KOR-cre* terminal immunoreactivity in lamina I or lamina III-V, and less dense in lamina II (which largely corresponds to the substantia gelatinosa in the mouse). Thus, the design of several of these studies is such that it would be difficult to detect a change, if any, as a result of KOR agonist activity on presynaptic terminals because they would likely been have below the level of detection. Given that we can now use the *KOR-cre* allele as a tool to more selectively activate the KOR-expressing population, and can record from areas with the densest innervation, it provides a greater opportunity to examine the presynaptic effect of KOR agonists more thoroughly.

In addition to expression at the central terminals, KORs are expressed on the peripheral terminals of primary afferents and likely also affect detection and transmission of somatosensory information from peripheral tissues. Peripherally-restricted or locally administered KOR agonists allow for the investigation of the contribution of peripheral KORs, but also allow for the ability to test the effect of activating peripheral KORs using modality selective stimuli in *in vivo* behavioral contexts. Previous studies have shown that systemically administered KOR agonists (Simonin et al., 1998); (Schepers, Mahoney, Gehrke, et al., 2008) or a locally administered KOR agonist (Vanderah et al., 2004) decrease sensitivity to noxious thermal stimuli in naïve mice, and locally administered KOR agonists decreased heat hyperalgesia in an inflammatory state (Cunha et al., 2012). Many studies have shown that peripherally-restricted or KOR agonists administered locally decrease mechanical hypersensitivity, measured by the paw pressure test, in

inflammatory states (Deuis et al., 2015); (Auh & Ro, 2012); (Binder et al., 2001); Machelska (Machelska et al., 1999); (Antonijevic et al., 1995); (Stein et al., 1989). One study has tested the effect of locally administered KOR agonists on mechanical hypersensitivity, measured by the von Frey test, and reported a decrease in mechanical threshold in an inflammatory state (Cunha et al., 2012). Alan Cowan and colleagues have shown previously that peripherally-restricted KOR agonists decrease scratching induced by histaminergic (Cowan et al., 2015) and non-histaminergic pruritogens (Inan & Cowan, 2004), supporting the idea that KOR-expressing primary afferents are involved in pruritoception as well as nociception. However, the effect of peripheral KOR agonists on the modulation of specific chemical algogens or pruritogens hasn't been fully examined, nor has the effect of KOR agonists on mechanical and thermal hyperalgesia been examined in a model of post-operative pain, which is a more clinically relevant model than the formalin or CFA assays that have been used previously. For the first time, we can use the characteristics of KOR-expressing DRG neurons to guide the investigation of KOR agonists' effects on behavioral responses. Therefore, we chose to use both preclinical studies with modality selective stimuli as well as a post-operative pain model because they provide an opportunity to investigate underlying mechanisms and provide a way to test for drugs that could be effective in similar clinical circumstances.

The experiments below were designed to investigate the effect of KOR activation at either the central or peripheral terminals of primary afferents, and to begin to ask how this affects the processing of information related to specific somatosensory modalities. First, experiments were performed to measure the effect of KOR agonists on neurotransmitter release from primary afferents in specific lamina in the spinal cord, with the prediction that KOR agonists would decrease glutamate release onto dorsal horn neurons in both laminae. Next, experiments were

conducted such that the contribution of central and peripheral KORs could be determined in assays that measure the behavioral responses to stimuli of different modalities. To do so, we used nalfurafine, a KOR agonist that would act on central and peripheral KORs, as well as two peripherally-restricted KOR agonists, ICI204,488 and FE200665. As KOR-expressing primary afferents expressed higher levels of multiple transcripts that have previously been shown to be involved in mediating thermal sensation and hyperalgesia (such as *Trpv1*, *Ptgir* and *Trka*), we predicted that peripherally-restricted KOR-agonists would decrease thermal withdrawal latencies at baseline and after injury. In addition, since KOR agonists showed a neurochemical profile consistent with their possible involvement in the transmission of chemical nociception and pruritoception, we predicted that KOR agonists would decrease behavioral responses to chemical algogens and pruritogens. Optogenetics were also used to test the behavioral responses to activation of *KOR-cre* primary afferents to determine if stimulation of these fibers would lead to a nocifensive and / or pruritoceptive response.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Mice were given free access to food and water and housed under standard laboratory conditions. The use of animals was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. KOR-cre mice were generated as previously described and are maintained on a mixed C57bl/6.129J background (Cai et al., 2016). For electrophysiology experiments, KOR-cre mice were mated with C57bl/6 mice or with Ai32 cre-responsive ChR2-

eYFP reporter mice (The Jackson Laboratory). Unless otherwise noted, 5 - 8 week old male and female C57bl/6 mice were used in behavioral experiments. To generate wild-type and KOR-cre-/- mice for behavioral and immunohistochemical analyses, heterozygous KOR-cre-/+ mice were harem mated and age-matched wild-type and KOR-cre-/- male and female offspring from the resulting litters were used.

3.2.2 Electrophysiology

Mice (4–7 weeks old) were deeply anesthetized and perfused transcardially with ice-cold oxygenated (95% O2 and 5% CO2) sucrose-based artificial cerebrospinal fluid (ACSF) (in mM; 234 sucrose, 2.5 KCl, 0.5 CaCl2, 10 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 11 Glucose). The thoracolumbar spinal cord was tranferred into a small chamber containing ice-cold oxygenated sucrose-based ACSF. For lamina I recordings, the dural and pial membranes were carefully removed and the spinal cord was pinned onto a Sylgard block with the right dorsal horn facing upward with the recording chamber and transferred to the rig. Then, a single parasaggital cut was made using a vibratome (Leica) to expose the gray matter, but not cut the dorsal root entry zone, then the spinal cord was pinned onto the Sylgard block with the sectioned surface facing upward in the recording chamber. For both recording types, the preparation was perfused with normal ACSF solution (in mM; 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3, 11 glucose) saturated with 95% O2 and 5% CO2 at 32˚C. Tissue was rinsed with ACSF for at least 30 min prior to recordings to wash out sucrose.

Neurons were visualized using a fixed stage upright microscope (BX51WI Olympus microscope) equipped with a 40x water immersion objective lens, a CCD camera (ORCA-ER Hamamatsu Photonics; XM10-IR, Olympus) and monitor screen. A narrow beam infrared LED

(L850D-06 Marubeni, emission peak, 850 nm) was positioned outside the solution meniscus, as previously described (Hachisuka et al., 2016). Whole-cell patch-clamp recordings were made with a pipette constructed from thin-walled single-filamented borosilicate glass and were filled with an intracellular solution containing the following (in mM): 135 K-gluconate, 5 KCl, 0.5 CaCl2, 5 EGTA, 5 HEPES, 5 MgATP, pH 7.2. Signals were acquired with an amplifier (Axopatch 200B, Molecular Devices). The data were low-pass filtered at 2 kHz and digitized at 10 kHz with an A/D converter (Digidata 1322A, Molecular Devices; Power1401-3, CED) and stored using a data acquisition program (Clampex version 10, Molecular Devices or Signal 6, CED). The liquid junction potential was not corrected. Cell recordings were made in voltage-clamp mode at holding potentials of -70mV to record excitatory postsynaptic currents (EPSCs). Blue light (GFP filter, centered around 485 nm, Lambda DG-4, Sutter instruments) was applied through the objective lens (x40) of the microscope. Light power on the sample was 1.3 mW/mm-2. The shutter was controlled by Clampex or Signal 6 software. Dynorphin A (Tocris Bioscience, 3195) and nor-BNI (Tocris Bioscience, 0347) were dissolved in ACSF and applied by exchanging solutions via a three-way stopcock.

3.2.3 Behavioral Assays

All assays were performed and scored by an experimenter blind to treatment and/or genotype. Acute itch and chemical pain assays were videotaped, and subsequently scored by an experimenter blind to treatment and/or genotype. Mice were pretreated with vehicle (sterile saline; 100µl IP), nalfurafine (20µg/kg; 100µl i.p.; Adooq Bioscience), ICI 204,488 (10mg/kg; 100µl i.p.; Tocris Bioscience), FE200665 (12mg/kg; 100µl i.p.; provided by YeonSun Lee and

Frank Porecca) 15 minutes prior to testing. All testing was performed in the University of Pittsburgh Rodent Behavior Analysis Core.

Optogenetic withdrawal assay

Mice were placed in a clear plastic container on an elevated wire grid and allowed to acclimate for 60 min. The plantar surface of the hindpaw was stimulated with a targeted blue light LED beam (coolLED pE-100) for 1 s at either 1 mW, 3 mW, or 10 mW intensity. The number of responses out of ten applications per intensity was recorded. Both KOR-cre-/- mice and their wild-type littermates received an i.p. injection of AAV9.FLEX.ChR2-tdTomato.SVRE.WP40 at P1. KOR-cre, RosalslChR2-eYFP mice did not receive an injection.

Acute itch behavior

The nape of the neck area was shaved at least twenty-four hours prior to the start of the experiment. Mice were placed in a clear plastic container (3.5” x 3.5” x 5”) for observation and allowed to acclimate for 30 min. An intradermal injection of chloroquine (200µg/20µl) was made into the nape of the neck. The number of scratch bouts was counted during a thirty-minute observation period.

Acute chemical pain behavior

Mice were placed in a clear plastic container for observation and allowed to acclimate for 30 min. After acclimatization, an intraplantar (i.pl.) injection of capsaicin (1.5%, 20ul) or acetic acid (0.6%, 20ul). The amount of time spent licking the injected paw was quantified during a ten- or thirty-minute period, respectively.

Hargreaves’ test

Mice were placed in a clear plastic container on a glass floor maintained at 30°C and allowed to acclimate for 60 min. A radiant heat beam was then focused onto the hind paw. The

latency to hindpaw withdrawal was recorded with 3 trials per animal. Beam intensity was adjusted so that at baseline mice displayed a latency of 8 - 12 s. A cut-off latency of 30 s was set to avoid tissue damage.

von Frey test

Mice were placed in a clear plastic container on an elevated wire grid and allowed to acclimate for 60 min. The plantar surface of the hind paw was stimulated with a set of calibrated von Frey filaments (0.008 - 6 g). The 50% paw withdrawal threshold was determined using the SUDO method (Bonin et al., 2014).

Post-operative pain assay

This assay was performed based on the original description in Brennan et al. (Brennan, Vandermeulen, & Gebhart, 1996) but adapted for use in mice as described in Pogatzki & Raja, (Pogatzki & Raja, 2003). Mice were anesthetized (2.0% for induction and 1.5% for maintenance) with isoflurane in a flow of O2, placed in a prone position on a heating pad covered with a blue fiber disposable towel. Laboratory tape was used to secure the left hind paw, which was then sterilized with betadine solution and wiped clean with sterile gauze. A 5 mm incision extending from the proximal edge of the heel towards the toes was made with a no. 11 scalpel. The incision was made through the glabrous skin, fascia and underlying plantaris muscle (leaving muscle origin intact), taking care not to cut the underlying ligaments. A single nylon mattress suture was used to close the wound and antibiotic ointment was applied to the area of the incision. Mice recovered from aneasthesia within 5 min and were returned to their home cage. Two and twenty-four hours after the incision, mice were placed in clear plastic containers either on an elevated wire grid for von Frey testing or on a glass floor for Hargreaves' testing. Mechanical sensitivity was recorded as the number of withdrawal responses (out of 10) to a single von Frey fiber (vF #

3.61, 0.4g) applied to the plantar surface of the paw adjacent to the incision. Thermal sensitivity was measured using the Hargreaves' test as described above, but the radiant heat beam was focused on the hindpaw near the area of the incision. Baseline measurements were made for both the von Frey and Hargreaves' tests twenty-four hours prior to the incision. Mice with wound dehiscence or puss around the incision at the time of behavioral testing were removed from the experiment.

3.3 RESULTS

3.3.1 A KOR agonist decreases glutamate release from primary afferents in the superficial and deep dorsal horn

In an attempt to address the function of KOR on primary afferents, first the effect of KOR activation at the central terminals in the spinal dorsal horn was investigated. As *KOR-cre* terminals were most dense in the very superficial dorsal horn and in the deeper dorsal horn, and less dense in the lamina propria, dorsal horn neurons in either lamina I or lamina III were targeted in the following experiments. To address the effect that KOR activation had on somatosensory transmission from primary afferents to second order neurons in the spinal cord, an experiment was designed that would measure the effects of KOR agonist dynorphin on the amplitude of light-evoked excitatory post-synaptic potentials (eEPSCs) observed upon optogenetic activation of *KOR-cre* afferents. Optogenetic stimulation was chosen over electrical root stimulation because selectively stimulating a smaller population of primary afferents that

were more likely to express KOR allowed for increased likelihood of detecting the effect of dynorphin.

To confirm the role of KOR on the central terminals of peptidergic afferents, the *ex-vivo* spinal preparation was used, which facilitates visualization of lamina I neurons for targeted whole cell patch clamp recordings (Figure 14A) (Hachisuka et al., 2016). Further, an advantage of this preparation is that it allows for an increased maintenance of spinal cord circuitry, and hence data collected are representative of a more complete dorsal horn environment than that collected using spinal cord slices. First, lamina I neurons that likely received monosynaptic input from *KOR-cre* afferents were identified based on eEPSC latency and absence of failure following optogenetic stimulation of *KOR-cre* afferent input. Next, paired-pulse blue light stimulation was applied through the microscope objective and these recordings were used to analyze the effect of dynorphin. These experiments revealed that dynorphin significantly and reversibly decreased the first peak eEPSC amplitude and increased the paired-pulse ratio that was observed upon optogenetic stimulation of *KOR-cre* afferents (Figures 14B, 14C and 14D). Similar results were observed when the experiments were repeated in the presence of naltrexone (1 μm), indicating that they were not due to a dynorphin-induced activation of MOR (Figures 15A, 15B and 15C). These findings suggest KOR activation reduces glutamate release from the central terminals of *KOR-cre* primary afferents in the superficial dorsal horn.

To investigate whether the *KOR-cre* afferents that target the deep dorsal horn were similarly modulated by KOR signaling, a modified *ex-vivo* spinal cord preparation was developed by Yu Omori in which a single parasagittal cut in one hemisphere of the spinal cord was performed in order to target neurons in LIII for patch-clamp recordings (Figure 14E). For these experiments, optogenetic stimulation of the dorsal root was performed to stimulate *KOR-*

cre positive afferents. To identify neurons in the dorsal horn that likely received monosynaptic input from these fibers, the absence of failure to elicit an EPSC following optogenetic stimulation of *KOR-cre* afferent input was used. Just as was found in lamina I, treatment with dynorphin caused a significant and reversible decrease in the first peak amplitude of eEPSCs, which corresponded to a significant increase in the paired-pulse ratio (Figures 14F, 14G and 14H). Similar results were also obtained in the presence of naltrexone (1 μm) to block MOR (Figures 15D, 15E and 15F). These findings suggest that *KOR-cre* afferents that target the deep dorsal horn (possibly including LTMRs) also show reduced glutamate release upon activation of KOR. Thus, KORs on the central terminals of primary afferents mediate presynaptic inhibition in both the superficial and deep dorsal horn.

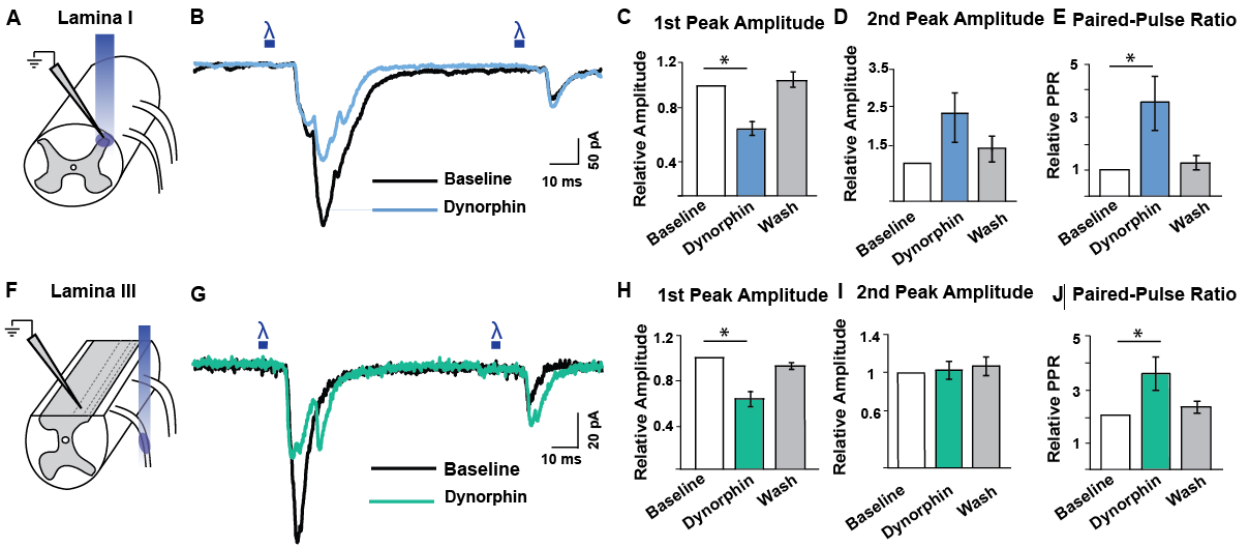


Figure 14. KOR activation reduces glutamate release from primary afferents that target lamina I and lamina III

A. Schematic illustrating the experimental set-up used to record from lamina I dorsal horn neurons while optogenetically stimulating *KOR-cre* primary afferent terminals. *KOR-cre* primary afferents were infected with a

Cre-dependent virus encoding channel rhodopsin (AAV.FLEX.ChR2-tdTomato; IP, P1) enabling selective activation of *KOR-cre* primary afferent terminals upon application of blue light to the spinal cord.

B. Representative trace of whole-cell patch clamp recording from a lamina I neuron at baseline (black) and during bath application of dynorphin (1 μM; blue). Two 5 ms pulses of blue light were given 100 ms apart to elicit light-evoked EPSCs.

C - E. Quantification of the relative amplitude of the first eEPSC (C), the relative amplitude of the second eEPSC (D) and paired-pulse ratio (E) at baseline (white), upon application of dynorphin (1 μM; blue), and following wash (gray). Dynorphin caused a significant decrease in the amplitude of the first eEPSC compared to baseline in lamina I neurons and a significant increase in the paired-pulse ratio compared to baseline, which was reversible (One-way ANOVA and Dunnett's multiple comparison's test, $p < 0.05$; $n = 14$ cells). Data are presented as mean ± SEM. There was no significant change in the 2nd peak amplitude during dynorphin application compared to baseline ($p = 0.1$; data not shown)

F. Schematic illustrating the experimental set-up used to record from lamina III dorsal horn neurons while optogenetically activating *KOR-cre* primary afferents. *KOR-cre, ROSA$^{lslChR2\text{-}eYFP}$* mice were used for these experiments, and therefore blue light stimulation was applied to the dorsal root in order to selectively activate *KOR-cre* primary afferents and not KOR-expressing spinal neurons.

G. Representative trace of whole-cell patch clamp recording from a lamina III neuron at baseline (black) and during bath application of dynorphin (1 μM; green). Two 5 ms pulses of blue light were given 100 ms apart to elicit light-evoked EPSCs.

H - J. Quantification of the first eEPSC relative amplitude (H), second eEPSC relative amplitude (I)and paired pulse ratio (J) at baseline (white), upon application of dynorphin (1 μM; green), and following wash (gray). Dynorphin caused a significant decrease in the amplitude of the first eEPSC and a significant increase in the paired-pulse ratio compared to baseline in lamina III neurons, which was reversible (One-way ANOVA and Dunnett's multiple comparison's test, $p < 0.05$; $n = 6$ cells). Data are presented as mean ± SEM. There was no significant change in the 2nd peak amplitude during dynorphin application compared to baseline ($p = 0.8$, data not shown).

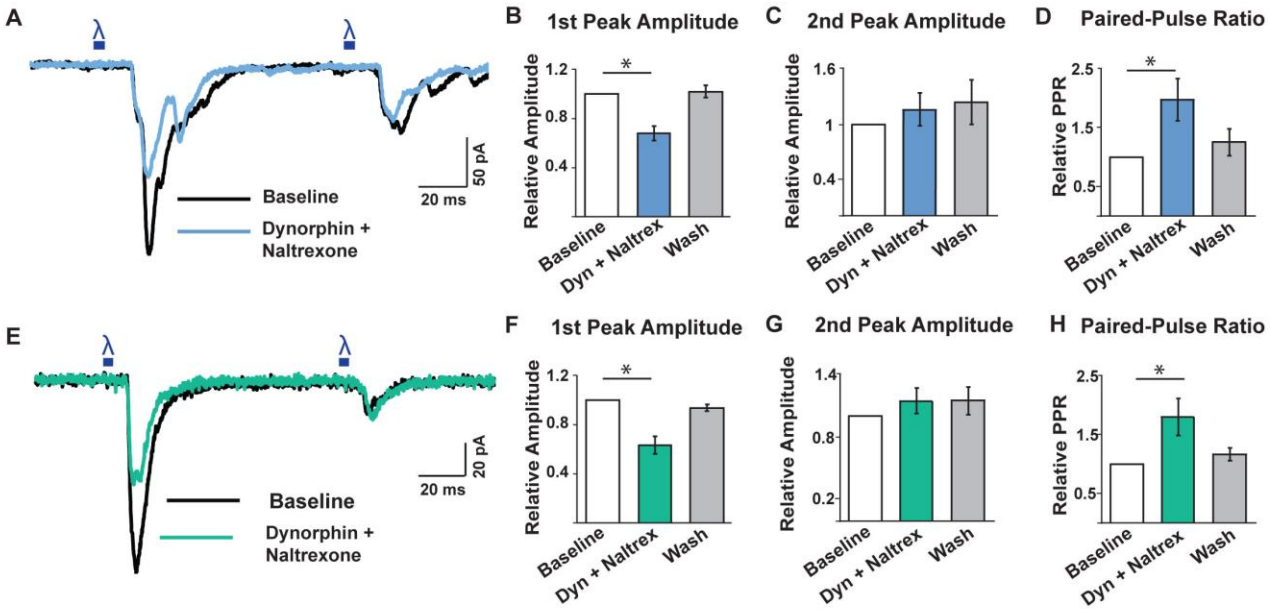


Figure 15. Dynorphin-mediated inhibition of glutamate release from KOR-expressing afferents is not inhibited by naltrexone

A. Representative trace of whole-cell patch clamp recording from a lamina I neuron at baseline (black) and during bath application of dynorphin (1 μm) + naltrexone (1 μm) (blue). Two 5 ms pulses of blue light were given 100 ms apart to elicit light-evoked EPSCs.

B - D. Quantification of the 1st eEPSC relative amplitude, 2nd eEPSC relative amplitude, and relative paired pulse ratio during baseline (white), drug (blue), and wash (gray) conditions in recordings from lamina I dorsal horn neurons following blue light stimulation of *KOR-cre* positive primary afferent terminals in *KOR-cre* + FLEX.ChR2 IP at P1. There was a significant decrease in 1st peak amplitude during dynorphin + naltrexone application compared to baseline in lamina I neurons (B). There was no significant change in 2nd peak amplitude during dynorphin + naltrexone application compared to baseline in lamina I neurons (C). There was a significant increase in the paired pulse ratio during dynorphin + naltrexone application compared to baseline in lamina I neurons (D). Data are presented as mean ± SEM. * $p < 0.05$, one-way ANOVA followed by Dunnett's multiple comparison's test, $n = 15$ cells.

E. Representative trace of whole-cell patch clamp recording from a lamina III neuron at baseline (black) and during bath application of dynorphin (1 μm) + naltrexone (1 μm) (green). Two 5 ms pulses of blue light were given 100 ms apart to elicit light-evoked EPSCs.

F - H. Quantification of the 1st eEPSC relative amplitude, 2nd eEPSC relative amplitude, and relative paired pulse ratio during baseline (white), drug (green), and wash (gray) conditions in recordings from lamina III dorsal horn neurons following blue light stimulation of the dorsal root in *KOR-cre Rosa*$^{lslChR2\text{-}eYFP}$ mice. There was a significant decrease in 1st peak amplitude during dynorphin + naltrexone application compared to baseline in lamina III neurons (F). There was no significant change in 2nd peak amplitude during dynorphin + naltrexone application compared to baseline in lamina III neurons (G). There was a significant increase in the paired pulse ratio during dynorphin + naltrexone application compared to baseline in lamina III neurons (G) Data are presented as mean ± SEM. * $p < 0.05$, one-way ANOVA followed by Dunnett's multiple comparison's test, n = 6 cells.

3.3.2 Optogenetic activation of *KOR-cre* primary afferents elicits withdrawal

Given the peptidergic nature of many KOR-expressing afferents, it seemed likely that selective activation of this population might elicit nocifensive withdrawal behaviors. To test this idea directly, channelrhodopsin 2 (ChR2) was expressed in *KOR-cre* mice using either a virus (FLEX.ChR2-tdt) or the Ai32 allele (Figure 16A). Blue light LED stimulation (470 nm; 1 s) was applied to the glabrous hind paw skin at one of three intensities (1 mW, 3 mW, or 10 mW). In control littermates (that had received the Cre-dependent virus but lacked *KOR-cre*), blue light stimulation did not elicit a withdrawal response at any intensity tested (Figure 16B; black line). In contrast, when ChR2 was expressed in *KOR-cre* afferents using either a virus or an allele, 3mW and 10mW light stimulation of the hind paw elicited a robust withdrawal response that was frequently accompanied by either jumping or licking/biting of the hind paw, or both (Figure 16B; blue lines and bars). These findings reveal that blue light induced activation of ChR2-expressing *KOR-cre* afferents causes a nocifensive withdrawal response.

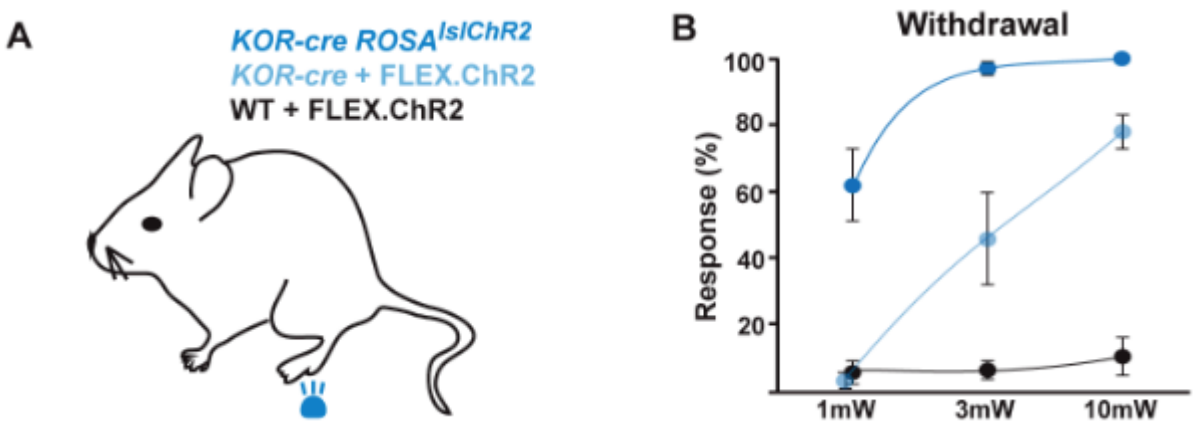


Figure 16. Optogenetic activation of *KOR-cre* afferents cause nocifensive withdrawal behavior

A. Schematic of optogenetic withdrawal experimental design. Blue light (470 nm) LED stimulation was applied to the glabrous hindpaw for 1 sec. Three different groups were tested; *KOR-cre; ROSA*^{lslChR2} mice, *KOR-cre* mice + FLEX.ChR2 (IP at P1), and the wild-type littermates (WT) + FLEX.ChR2 (IP at P1).

B. The percentage of mice showing withdrawal upon optogenetic stimulation at three different LED intensities (1 mW, 3 mW, and 10 mW). Data are presented as mean ± SEM (n = 4 - 6 mice/group).

3.3.3 Peripherally-restricted KOR agonists inhibit chemical pain and itch behaviors, but no thermal thresholds

Given that activation of KOR-expressing afferents appeared to be aversive, we next investigated whether inhibition of these cells with a KOR agonist would reduce behavioral responses to noxious or pruritic stimuli. To do so, we compared the effects of nalfurafine, a centrally-penetrating KOR agonist (Endoh et al., 2001); (Nagase et al., 1998), to those of either ICI204,488 or FE200665, two peripherally restricted KOR agonists that have extremely limited ability to cross the blood brain barrier (Figure 7C) (Shaw, Carroll, Alcock, & Main, 1989); (Vanderah et al., 2008). For these experiments, mice of both sexes were used and, since no difference between sexes was observed, data were pooled.

Following an intraplantar (IPL) injection of capsaicin (20 μL of 1.5% capsaicin), mice treated with any of the three KOR agonists showed a significant decrease in the time spent licking the injected hind paw (Figure 17A) compared to mice treated with vehicle. Similarly, all three KOR agonists reduced the time that mice spent licking the injected hind paw following an IPL injection of acetic acid (20 μL of 0.6% AA; Figure 17C). Furthermore, both centrally active and peripherally-restricted KOR agonists reduced scratching behavior in response to intradermal chloroquine (200 μg in 20 μL; Figure 17D). Importantly, these effects were due to the action at KOR, rather than an off-target effect of the drugs. To test the specificity of these agonists, we tested their effects in homozygous KOR-cre knockin mice (i.e. KOR -/-). We found that nalfurafine decreased chloroquine-induced scratching in wild-type littermates but not in KOR-cre homozygous mice (Figure 18A). Further, we observed that nalfurafine increased paw withdrawal latency (PWL) in the Hargreaves test in wild-type littermates but not in KOR-cre homozygous mice (18B). Similarly, we observed that ICI204,448 or FE200665 did not decrease chloroquine-induced scratching in mice that lacked KOR (Figures 18C - 18D), but did significantly reduce chloroquine-induced scratching in wild-type littermates. Taken together, these results suggest that peripherally-restricted KOR agonists are sufficient to decrease acute pain- and itch-related behaviors caused by chemical irritants.

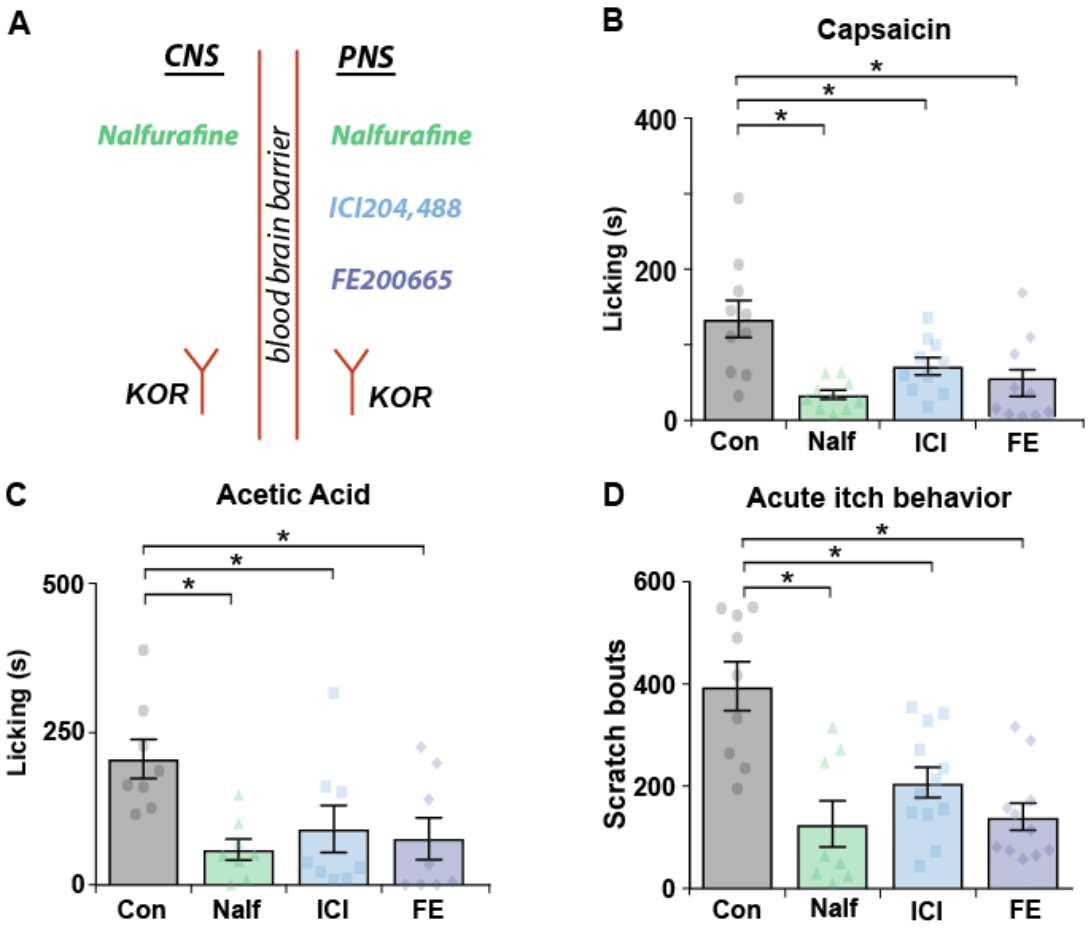


Figure 17. Peripherally-restricted KOR agonists decrease chemical pain and itch

A. Schematic diagram illustrating kappa agonists used in behavioral assays in D - J. Nalfurafine acts both centrally and peripherally, whereas ICI204,488 and FE200665 are peripherally restricted KOR agonists. Nalfurafine (Nalf; 200 µg/kg), ICI204,448 (ICI; 10 mg/kg), and FE200665 (FE; 12 mg/kg) were given IP 15 minutes prior to behavioral testing. All data are presented as mean ± SEM and colored shapes represent data points from individual animals.

B. Capsaicin-induced licking behavior (20 µL, intraplantar, 1.5%) was significantly reduced by Nalf, ICI, or FE (One-way ANOVA with Dunnett's multiple comparison's test, $p < 0.001$; n = 10 mice/group).

C. Acetic Acid-induced licking behavior (20 µL intraplantar, 0.6%) was significantly reduced by Nalf, ICI, or FE (One-way ANOVA with Dunnett's multiple comparison's test, $p < 0.05$; n = 10 mice/group).

D. Chloroquine-induced scratching behavior (20 µL intradermal, 200 µg) was significantly reduced by Nalf, ICI, or FE (One-way ANOVA with Dunnett's multiple comparison's test, $p < 0.001$; n = 8 - 12 mice/group).

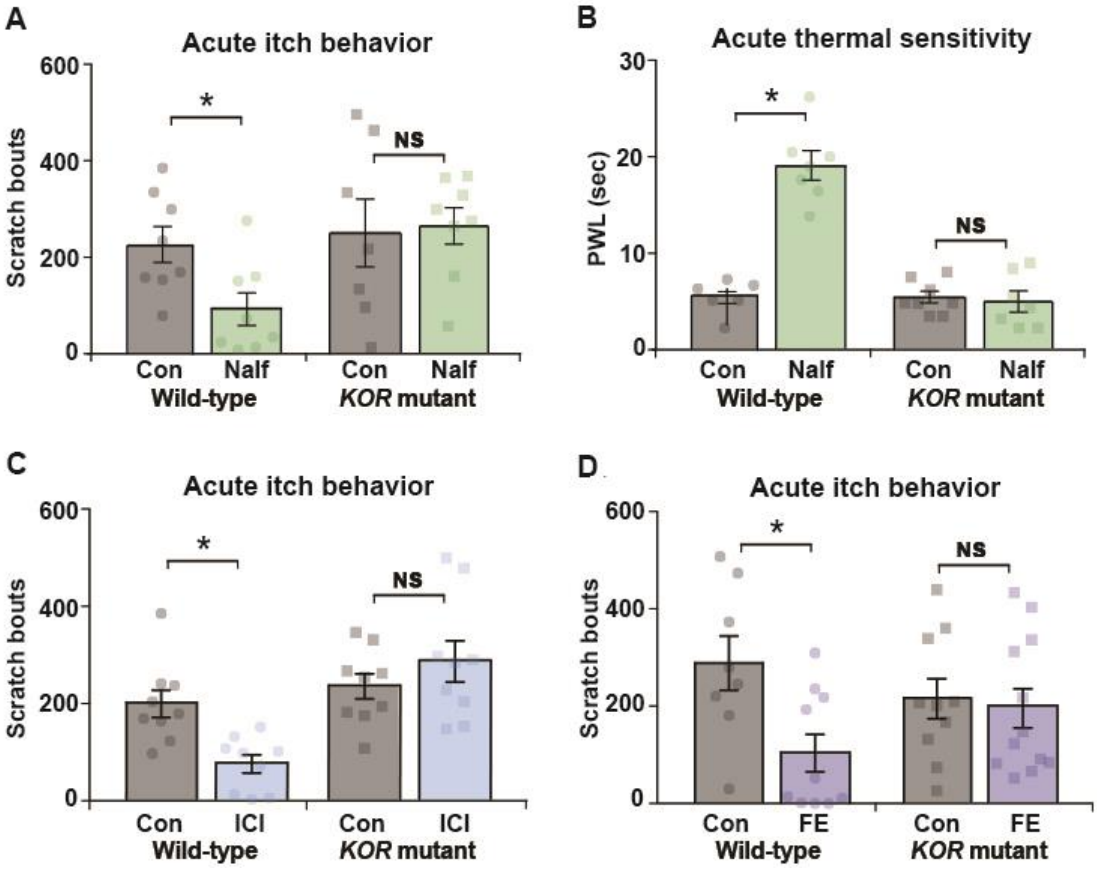


Figure 18. The effects of nalfurafine, ICI204,488 and FE200665 on itch and pain behaviors are specific to KOR

Nalfurafine (Nalf, 200 μg/kg), ICI204,488 (ICI, 10 mg/kg), or FE200665 (FE, 12 mg/kg) were given IP 15 minutes prior to the start of the behavioral assays. *KOR* mutant mice, which are homozygous for the *KOR-cre* allele, and their wild-type littermates were used for these experiments. All data are presented as mean ± SEM and colored shapes represent data points from individual mice.

A. Nalfurafine significantly decreased chloroquine-induced scratching behavior (20 μl intradermal, 200 μg) in wild-type mice (*, $p < 0.05$, two-way ANOVA with General Linear Hypothesis Test). In *KOR* mutant mice, nalfurafine had no significant effect (NS, $p > 0.05$). n = 7 – 8 mice/group.

B. Nalfurafine significantly paw withdrawal latency to thermal heat (Hargreaves' test) in wild-type mice, (*, $p < 0.001$, two-way ANOVA with General Linear Hypothesis Test). In *KOR* mutant mice, nalfurafine had no significant effect (NS, $p > 0.05$). n = 7 – 8 mice/group.

C. ICI204,448 significantly decreased chloroquine-induced scratching behavior (20 µl intradermal, 200 µg) in wild-type mice (*, $p < 0.001$, two-way ANOVA with General Linear Hypothesis Test,). In *KOR* mutant mice, ICI204,448 had no significant effect (NS, $p > 0.05$). n = 9 mice/group.

D. FE200665 significantly decreased chloroquine-induced scratching behavior (20 µl intradermal, 200 µg) in wild-type mice, but not in *KOR* mutant mice (*, $p < 0.01$, two-way ANOVA with General Linear Hypothesis Test,). In *KOR* mutant mice, ICI204,448 had no significant effect (NS, $p > 0.05$). n = 8 – 12 mice /group.

To determine the modality selectivity of the responses to peripherally-restricted KOR agonists, their effects on mechanical and thermal sensitivity were investigated, both under naïve conditions and in a model of post-operative pain. In naïve mice, none of the KOR agonists influenced paw withdrawal threshold to the application of von Frey filaments (Figure 19A). Furthermore, only nalfurafine, but not the peripherally restricted agonists ICI204,448 or FE200665, inhibited thermal responsivity in the Hargreaves' assay (Figure 19B).

Following injury, such as an incision during surgery, nociceptor activation and/or sensitization (Pogatzki, Vandermeulen, & Brennan, 2002); (Brennan, 2011) can drive ongoing pain and hypersensitivity to mechanical and thermal stimuli (Brennan et al., 1996). Analyzing the effect of injection of a receptor-specific ligand (such as the assays used in Figure 17), while useful to study specific mechanisms, does not recapitulate the etiology and development of tissue conditions causing pain following an incision. Therefore, the Brennan incision model (Brennan et al., 1996) modified for use in mice (Pogatzki & Raja, 2003) was used in an effort to investigate whether KOR agonists might be useful for the management of post-operative pain. With this model, we found that nalfurafine, ICI204,488, or FE200665 inhibited mechanical hypersensitivity at two hours following incision of the hind paw (Figure 19C), but not at twenty-four hours following incision (Figure 19E). Only nalfurafine, but not the peripherally restricted

agonists, inhibited thermal hypersensitivity in the Brennan model (Figure 19D and 19F) at two hours and twenty-four hours following incision. Whereas nalfurafine attenuated both thermal and mechanical hypersensitivity, peripherally-restricted KOR agonists were only sufficient to inhibit acute mechanical hypersensitivity.

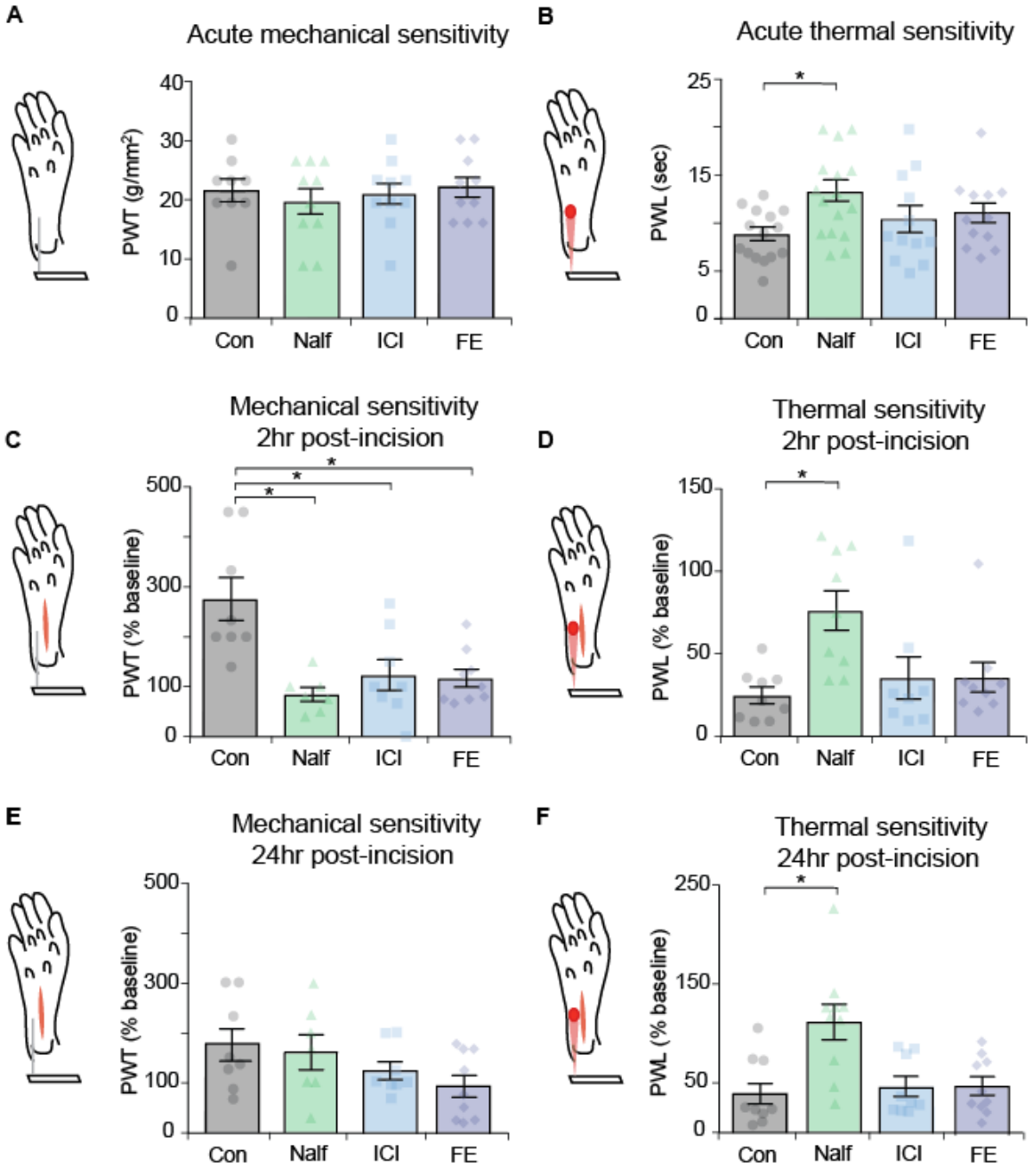


Figure 19. Peripherally-restricted KOR agonists decrease acute mechanical hypersensitivity following incision, but not thermal thresholds

A. Paw withdrawal threshold (PWT) as measured by the von Frey test was not significantly changed by Nalf, ICI, or FE (One-way ANOVA, $p = 0.8$; $n = 9$ - 10 mice/group).

B. Nalfurafine significantly increased paw withdrawal latency (PWL) as measured by the Hargreaves' test, but ICI and FE had no effect on PWL (One-way ANOVA with Dunnett's multiple comparison's test (* $p < 0.05$; $n = 12$ - 16 mice/group).

C. Mechanical hypersensitivity, measured 2 hours after an incision of the hind paw, was significantly reduced by Nalf, ICI, or FE (One-way ANOVA with Dunnett's multiple comparisons test, *, $p < 0.001$; $n = 7 – 9$ mice/group). Mechanical sensitivity was recorded as the number of withdrawal responses (out of 10) to a single von Frey fiber (vF # 3.61, 0.4g) applied to the plantar surface of the paw adjacent to the incision. Data are normalized to a baseline measure recorded 24 hours prior to the incision.

D. Thermal hypersensitivity, measured 2 hours after an incision of the hindpaw, was significantly reduced by Nalf (One-way ANOVA with Dunnett's multiple comparison's test, *, $p < 0.01$), whereas ICI or FE had no significant effect ($p > 0.05$; $n = 8 – 9$ mice/group). Data are normalized to a baseline measure recorded 24 hours prior to the incision.

E. Mechanical hypersensitivity, measured 24 hours after an incision of the hind paw, was not significantly changed by Nalf, ICI, or FE (One-way ANOVA with Dunnett's multiple comparison's test, *, $p = 0.14$; $n = 7 – 9$ mice/group). Data are normalized to a baseline measure recorded 24 hours prior to the incision.

F. Thermal hypersensitivity, measured 24 hours after an incision of the hindpaw, was significantly reduced by Nalf (One-way ANOVA with Dunnett's multiple comparison's test, $p < 0.05$), whereas ICI or FE had no significant effect ($p > 0.05$; $n = 8 – 9$ mice/group). Data are normalized to a baseline measure recorded 24 hours prior to the incision.

3.4 DISCUSSION

The experiments within this chapter were aimed at determining how KOR agonists affect somatosensory transmission at primary afferent central terminals and at the level of the periphery. Whole-cell patch clamp recordings in dorsal horn neurons in both lamina I and lamina III demonstrated that KOR agonists could decrease glutamate release from primary afferent fibers. Activation of *KOR-cre* afferents using optogenetics induced a withdrawal response consistent with pain sensation supporting the idea that these afferents are involved in transmitting nociceptive information. Complementary results using KOR agonists to decrease activity of KOR-expressing afferents (instead of using optogenetics to activate KOR-expressing afferents) showed a decrease in pain- and itch-related behaviors. Behavioral assays showed that peripheral KOR agonists were sufficient to decrease pain- and itch-related behaviors caused by chemical irritants, while peripherally and centrally acting KOR agonists decreased mechanical hypersensitivity in a model of post-operative pain. Surprisingly, only a KOR agonist that penetrates the blood-brain barrier, nalfurafine, decreased noxious thermal sensitivity either in naïve mice or in a model of post-operative pain. These data suggest that there are multiple sites of action for KOR agonists (endogenous or exogenous) to modulate somatosensory information, and support further exploration of peripherally-restricted KOR agonists in the treatment of pain and itch conditions.

In contrast to several other publications that did not observe an effect of KOR agonists on primary afferent release of neurotransmitter into the spinal cord (Ikoma et al., 2007); (Kohno et al., 1999); (Ueda et al., 1995), we observed a KOR agonist-mediated decrease in evoked EPSC amplitude in dorsal horn neurons upon optogenetic stimulation of *KOR-cre* afferents. As mentioned in the introduction of this chapter, our characterization of KOR-expressing primary

afferent allowed for more specific methodological design that would give us an advantage to detect this phenomenon, if it was indeed occurring. To the best of our knowledge, this is the first time this effect has been reported in both the superficial and deeper dorsal horn. Terminals in these different laminae are likely to be functionally distinct in that afferents targeting lamina I are more likely to transmit nociceptive information and those in deeper lamina (II-V) are more likely to transmit low-threshold mechanosensation. However, this conclusion cannot be definitely reached without more specific experiments (see Section 4.6 Future Directions).

Based on the effects of peripherally-selective KOR agonists, it might have been expected that optogenetic activation of *KOR-cre* afferents would result in both pain and itch behaviors. However, we observed withdrawal behaviors that are more consistent with nocifensive withdrawal than itch-related behaviors. Because pain generally inhibits itch (for review, see (Schmelz, 2005)), an alternative possibility is that itch might be occluded upon co-activation of *KOR-cre* nociceptors and pruritoceptors. In the experiment described above, activation of *KOR-cre* afferents that innervate the glabrous hind paw resulted in behaviors that are consistent with a nocifensive response (withdrawal, jumping and licking/biting behaviors). Alternatively, itch-associated behaviors may have been difficult detect since mice cannot scratch glabrous skin, which was the site of LED stimulation. Consistent with this possibility, when LED stimulation was targeted KOR-expressing afferents that innervate the ear, we observed abrupt withdrawal from the laser that was sometimes accompanied by ear-directed scratching with the hindlimb (personal observation), a behavior that is at least consistent with pruritoception. Since peripherally-restricted KOR agonists reduce chemical pruritogen-induced scratching, it is possible that a subset of KOR-expressing fibers that innervate the skin are involved in mediating itch; if so, however, it remains to be resolved which KOR-expressing afferents are involved.

In naïve mice, neither nalfurafine nor the peripherally-restricted KOR agonists affected mechanical threshold as measured by the von Frey test. Because the withdrawal threshold in the von Frey test is likely already at threshold, though, it is likely that it would be difficult to measure a KOR-mediated increase in mechanical threshold using this method. Future studies could investigate a KOR agonist driven effect on baseline mechanical sensitivity using a method such as the paw pressure test. The observation that the peripherally-restricted KOR agonists didn't affect mechanical threshold in this assay isn't overly surprising, as *KOR-cre* fibers did not overlap substantially with IB4-expressing C-fibers nor show overlap with A-fibers innervating Meissner's corpuscles or Merkel cells in the glabrous skin. Before conducting the experiment, we predicted that nalfurafine would have had an effect on mechanical sensitivity because a recent study reported that ablation of dynorphin-cre interneurons in the spinal cord led to an increase in sensitivity to static and dynamic mechanical sensitivity using von Frey and brush assays, respectively (Duan et al., 2014). The authors interpreted these results as a role for dynorphin-cre interneurons in the inhibition of low-threshold mechanosensation, but did not investigate whether this effect was due to release of dynorphin or GABA/glycine. The results here are consistent with the interpretation that perhaps dynorphin release in the spinal cord does not play a large role in low-threshold mechanical sensitivity, at least not in the glabrous skin, and the phenotype observed after ablation of dynorphin-cre interneurons may more heavily involve GABA/glycine signaling than dynorphin signaling. Although the KOR agonists did not affect mechanical sensitivity in the experiment described here, the von Frey test used is measuring low-threshold mechanical sensitivity. Future experiments could determine the effects of KOR agonists on mechanical sensitivity in the naïve state, specifically within the noxious range, by using a paw pressure assay. This type of assay would also likely involve afferents innervating

deeper tissues as well as cutaneous afferents, unlike the von Frey assay which activates cutaneous fibers.

Interestingly, nalfurafine and the peripherally-restricted KOR agonists did decrease mechanical hypersensitivity at 2 hours after an incision of the hind paw in the model of post-operative pain. A possible mechanism underlying this observation is that KOR-expressing afferents are sensitized following the incision and KOR agonists prevented or decreased the level of this sensitization. Primary afferent sensitization is defined as a gain of mechanical sensitivity, decreased threshold to mechanical stimuli, an increase in background activity, an increased responsiveness to the same stimuli, or an increase in the size of the receptive field. Brennan and colleagues have previously compared the responses of primary afferents to mechanical stimulation before and after incision. They report the primary mechanism of mechanical sensitization at 45 min post-incision observed across fiber types (Aδ, C fibers, or mechanically-insensitive fibers) was an expansion of the receptive field (Hamalainen, Gebhart, & Brennan, 2002). Thus, expansion of the receptive field of multiple fibers could cause an increase in the number of fibers activated during the presentation of a punctate mechanical stimulus. If a great enough proportion of these fibers express KOR, it could be that KOR agonists were sufficient to mitigate the resulting spatial summation and increased mechanical sensitivity. Alternatively, afferents responding to the mechanical stimuli before and after the incision could start expressing KOR after injury, and hence the KOR agonists could potentially decrease the responsiveness of these fibers. Consistent with this possibility, previous studies have shown an increase in KOR mRNA and protein levels in the DRG following injury (Caram-Salas et al., 2007); (Zambelli et al., 2014); (Obara et al., 2009); (Puehler et al., 2006). However, it is unclear if this increase was within primary afferents that already express the KOR, due to *de novo* expression in other

primary afferents, or both (future studies investigating this question will be considered in section 4.0 General Discussion).

In contrast to the mechanical sensitivity assay, nalfurafine did decrease thermal sensitivity in naïve mice as measured by the Hargreaves' test. The peripherally-restricted KOR agonists did not change thermal sensitivity suggesting the effect of nalfurafine was due to action at central KORs. The observation that the peripherally-restricted KOR agonists did not affect thermal sensitivity was surprising, as many KOR-cre positive afferents expressed TRPV1-IR and all *Oprk1*-expressing peptidergic afferents tested expressed *Trpv1* mRNA. TRPV1 is known to be activated by heat stimuli into the noxious range (Caterina et al., 1997), and ablation of TRPV1-expressing fibers decreases thermal sensitivity (Cavanaugh et al., 2009). However, the TRPV1 channel is known to be activated by other stimuli as well such as protons and vallinoid compounds like capsaicin (Caterina et al., 1997). Further, a larger proportion of TRPV1-expressing afferents target deeper tissues which are likely to be less involved in detecting noxious heat and may be more involved in detecting protons, vallinoids, or be a downstream target interacting with other membrane proteins (Christianson, McIlwrath, Koerber, & Davis, 2006). Therefore, the subset of TRPV1- and KOR-expressing afferents could be tuned to transmit information regarding the chemical milieu of the surrounding tissue rather than temperature.

Consistent with this interpretation, only nalfurafine decreases thermal hypersensitivity following an incision of the hind paw in the post-operative pain model. This suggests that the effect was driven by nalfurafine's action at central KORs, and that primary afferents expressing KOR are not greatly involved in transmitting information about noxious heat in a naïve state nor post-injury. This contrasts with results of previous studies investigating the effect of peripheral

KOR activation on thermal sensitivity either in naïve mice (Vanderah et al., 2004) or in an inflammatory state (Cunha et al., 2012). These studies gave local injections of KOR agonists, though, which could potentially underlie the difference. It could be that most of the KOR-expressing fibers are more tuned for chemical detection, but some of them do transmit temperature information and a local injection could be more effective at inhibiting these fibers, whereas a systemic injection of a peripherally-restricted agonist may not.

4.0 GENERAL DISCUSSION

4.1 OVERVIEW

This study expanded our knowledge of how the kappa opioid receptor system modulates somatosensory information and built a foundation for future exploration of this system as a potential target for the treatment of pain and itch conditions. The experiments described herein investigated the primary afferent cell types that express KOR through characterization of their anatomical, neurochemical, and transcriptional profiles using a combination of immunohistochemistry, viral tracing, and single cell RT-PCR in a *KOR-cre* mouse line. Building upon this information, additional experiments investigated if KOR agonists could influence somatosensory transmission either centrally, peripherally or both using either in vitro physiology or in vivo behavioral models.

We demonstrated that KOR is expressed on at least two distinct subtypes of primary afferent fibers that differ in both their anatomical and neurochemical profiles. One is a group of peptidergic putative nociceptors and/or pruritoceptors that express significantly higher levels of particular mRNA transcripts classically expressed in peptidergic afferents, and target multiple tissues throughout the body. Another is a group of putative myelinated LTMRs that form specialized lanceolate or circumferential endings around hair follicles in the skin. These two groups' central terminals targeted distinct lamina in the spinal dorsal horn, either the very

superficial lamina or the deeper dorsal horn, respectively. Further, using optogenetics, we found that KOR agonists decrease evoked EPSC amplitude resulting from optogenetic activation of KOR-expressing primary afferent terminals in both the superficial and deeper dorsal horn. Finally, we tested the effect of either a centrally and peripherally acting KOR agonists or peripherally restricted agonists in multiple behavioral models of pain and itch. Importantly, we demonstrated that each of the KOR agonists we used was specific to the KOR, as each drug had no effect in KOR homozygous mice. Peripherally-restricted KOR agonists decreased responses to chemical algogens and pruritogens, but only were sufficient to decrease mechanical, not thermal, hypersensitivity in a model of postoperative pain. In summary, these experiments define at least two subgroups of primary afferent neurons expressing KOR (Figure 8), and provide insight into how KOR agonists act to alter somatosensory transmission.

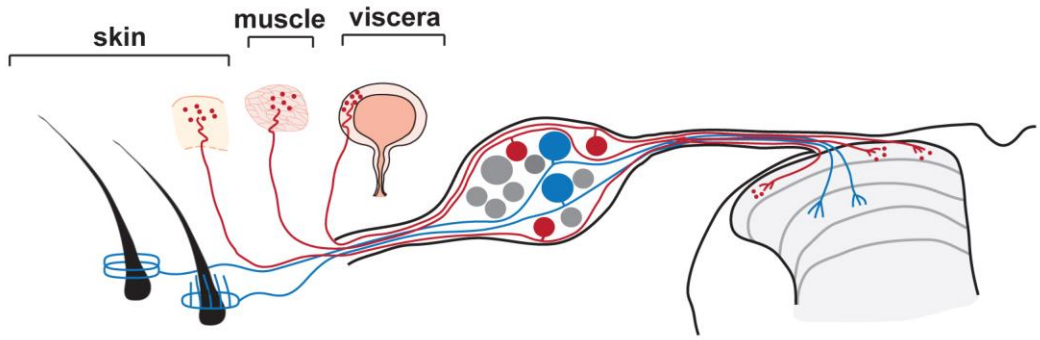


Figure 20. KOR is expressed by peptidergic afferents innervating multiple tissue types as well as a subset of LTMRs that target hair follicles

Schematic of the primary afferent subtypes that express the KOR. One is a subset of LTMRs that form lanceolate and circumferential endings around hair follicles in the skin (blue fibers). Another is a subset of peptidergic afferents (red fibers) that expresses particularly high transcript levels of classic peptidergic markers such as CGRP and Substance P, and these fibers innervate multiple tissue types including the skin, muscle, and viscera.

4.2 POSSIBLE MECHANISMS UNDERLYING KOR AGONISTS' EFFECTS IN A MODEL OF POST-OPERATIVE PAIN

The study we performed investigating KOR agonist effects on hypersensitivity to a mechanical or thermal stimulus demonstrated that peripherally-restricted KOR agonists decreased mechanical, but not thermal, hypersensitivity. Previous experiments measuring afferent activity in the incision model by Brennan and colleagues can give us some insight into the mechanism underlying this observation and inspire ideas for experiments to test future hypotheses. Skin incision alone has been shown to be sufficient to cause hypersensitivity to evoked behaviors caused by mechanical and thermal stimuli (J. Xu & Brennan, 2009). Even though incision of both the skin and deeper tissue was made, the experiments described herein involved behavioral assays that primarily stimulate cutaneous tissue. Due to the nature of stimulus application in the von Frey (and Hargreaves tests), we are only measuring the effect of KOR agonists on cutaneous afferents. Future experiments of interest include using an assay that would also stimulate deeper tissues, such as a paw pressure assay, which could test more specifically the effects of KOR agonists on pain-related behaviors caused by stimulation of superficial and deep tissues. Several studies have reported mechanical sensitization of muscular, visceral, or articular afferents after injury or in inflammatory states (Ustinova, Fraser, & Pezzone, 2006); (J. L. Ross, Queme, Shank, Hudgins, & Jankowski, 2014); (Schaible & Schmidt, 1985); (Neugebauer, Schaible, & Schmidt, 1989). As KOR-expressing primary afferents target multiple tissue types and not only cutaneous tissue, it is possible that peripherally-restricted KOR agonists could also decrease mechanical hypersensitivity in deeper tissues after injury as well.

After an incision of the hind paw in rats, Brennan and colleagues have shown an increase in spontaneous activity at the level of both the primary afferents and in dorsal horn neurons, that

wasn't observed until approximately one day following incision. The spontaneous activity in dorsal horn neurons following an incision is still largely dependent on primary afferent input, as bupivacaine treatment of the incised tissue decreased the level of spontaneous activity in dorsal horn neurons (J. Xu & Brennan, 2009); (Brennan, 2011). They have shown that deep tissue incision, compared to an incision of the skin alone, was necessary to induce spontaneous activity of both the primary afferents and dorsal horn neurons as well as spontaneous pain behaviors (J. Xu & Brennan, 2009); (J. Xu & Brennan, 2010). Therefore spontaneous activity of primary afferents, in particular cutaneous afferents, is likely not a major driver of hypersensitivity to evoked pain behaviors. Future experiments measuring both the effect KOR agonists have on spontaneous activity in primary afferents of deeper tissues and ongoing pain behaviors (in addition to evoked pain behaviors) following an incision would further inform their therapeutic potential in surgical procedures that incise deeper tissues. However, dilute capsaicin treatment after incision reduces heat hypersensitivity and spontaneous pain behaviors, but not mechanical hypersensitivity, suggesting these may be separate mechanisms (Kang, Wu, Banik, & Brennan, 2010). Brennan and colleagues have also reported that intraplantar injection of TrkA IgG or anti-NGF increased heat hyperalgesia and spontaneous pain behaviors, but did not change mechanical thresholds (Zahn, Subieta, Park, & Brennan, 2004); (Banik, Subieta, Wu, & Brennan, 2005). Since peripherally-restricted KOR agonists decreased mechanical hypersensitivity, but not heat hypersensitivity, one might predict that peripherally-restricted KOR agonists would also therefore not affect spontaneous pain behaviors.

Interestingly, in the experiments described herein, at two hours post-incision, peripheral KOR agonists were sufficient to cause a decrease in mechanical hypersensitivity, but the decrease after treatment with a peripheral KOR agonists was lost at twenty-four hours post-

incision. As spontaneous activity wasn't observed post-incision until one day later and not in skin incision alone, these data further support the conclusion that spontaneous activity wasn't the mechanism underlying evoked mechanical hypersensitivity to von Frey filaments. Other potential mechanisms underlying hypersensitivity of evoked behavioral responses are decreased response threshold of primary afferents, increased response of primary afferents, and / or an increase proportion of primary afferents responding to a given stimulus (Brennan, 2011; Hamalainen et al., 2002). Physiological recordings of primary afferents fibers in mice following an incision injury could measure the influence of peripheral KOR agonists on each of these parameters to define the mechanism(s) of action for the reduction of hypersensitivity to mechanical stimuli within the first hours following incision, particularly in cutaneous afferents. Experimental models in humans evaluating the mechanisms of sensitization following an incision have supported the idea that primary hypersensitivity (in the area of tissue damage) is mediated by primary afferent activity, but secondary hypersensitivity (outside the area of tissue damage) involves other mechanisms, such as central sensitization (Kawamata, Takahashi, et al., 2002), (Kawamata, Watanabe, et al., 2002). Therefore, if peripheral KOR agonists can decrease primary afferent responsiveness to evoked stimuli in the area of tissue injury, it is promising sign that these drugs may be effective at reducing hypersensitivity following incision.

An interesting and surprising observation made in the experiments using the post-operative pain model is the dissociation between peripherally-restricted KOR mitigation of mechanical and not thermal hypersensitivity, especially given the transcriptional profile of KOR-expressing neurons. The studies described here show that KOR-expressing primary afferents have higher levels of several transcripts that have been shown to be involved in heat pain and heat hyperalgesia, most noticeably TRPV1. In contrast to what one might predict, peripherally-

restricted KOR agonists did not have an effect on noxious heat thresholds. This, at first, was surprising because KOR agonists decreased behavioral responses to both capsaicin and acetic acid, which also have been shown to activate TRPV1. However, we favor the hypothesis that the subset of peptidergic neurons that express KOR and high transcript levels of *Trpv1* are tuned for detection of chemical ligands, rather than for noxious heat stimuli. Even though TRPV1 is most commonly associated with detecting and transmitting heat stimuli, this channel is also activated by protons, chemical ligands, and can be a transducer downstream of GPCRs (Caterina et al., 1997). This isn't to say that these KOR- and TRPV1-expressing neurons don't respond to heat stimuli at all, but their activity pattern and or connectivity pattern when presented with a heat stimulus *in vivo* may not be optimally tuned as a pattern that would lead to noxious heat sensation. Instead, activation of TRPV1 on these primary afferents by chemical stimuli may instead produce a pattern of activity or activation of a circuit that is more heavily involved in transmitting information regarding the chemical milieu of the tissue environment, and perhaps be more likely to be involved in generating a burning or stinging sensation. If this were the case, one might expect KOR agonists to decrease behavioral responses to chemical TRPV1 agonists that have been reported to cause a burning sensation (LaMotte, Shimada, & Sikand, 2011), but not to noxious heat.

Consistent with this idea is a study using the postoperative pain model that showed a decrease in tissue pH for several days following incision (Woo, Park, Subieta, & Brennan, 2004), indicating that tissue acidosis plays a role in nociception after an incision injury. A study by Steen et al. (Steen, Reeh, Anton, & Handwerker, 1992) in rats observed a population of cutaneous polymodal C-fibers that responded to low pH application to their receptive fields in the skin, and many of the fibers responding to protons also responded to capsaicin indicating

they expressed TRPV1. They reported that repeated or longer exposure low pH solutions sensitized polymodal C-fibers such that their discharge rate was increased and the von Frey thresholds were decreased, and this occurred regardless of whether the C-fibers originally responded to low pH solutions. These data are supportive of the idea that KOR-expressing fibers are activated and sensitized by molecules that could activate TRPV1 (such as protons) following an incision injury, and this could lead to a decrease in mechanical thresholds. KOR agonists decrease cAMP through inhibition of adenylyl cyclase (Bruchas & Chavkin, 2010). This would limit the phosphorylation of the TRPV1 channel by protein kinase A thereby allowing for desensitization of TRPV1 (Pierre et al., 2009). Ion influx and depolarization through TRPV1 channels can increase release of inflammatory neuropeptides, such as CGRP and SP (Planells-Cases, Garcia-Sanz, Morenilla-Palao, & Ferrer-Montiel, 2005). Thus, not only could KOR agonists prevent sensitization of KOR-expressing afferents, but they could decrease release of inflammatory neuropeptides that would sensitize other afferents (even those that did not respond to protons originally).

Why a KOR-mediated decrease in CGRP and/or SP release would not prevent the sensitization of afferents that are involved in heat hyperalgesia is unclear and cannot be resolved from the post-operative pain model experiments alone. In cutaneous afferents, studies have observed afferent sensitization to heat stimuli, but sensitization to mechanical stimuli after injury/inflammation in cutaneous afferents has been rarely observed (Andrew & Greenspan, 1999); (Banik & Brennan, 2009); (Kirchhoff, Jung, Reeh, & Handwerker, 1990); (Banik, Kozaki, Sato, Gera, & Mizumura, 2001); (Du, Zhou, & Carlton, 2006); (Schlegel, Sauer, Handwerker, & Reeh, 2004). This has led to the suggestion that mechanical hypersensitivity is primarily due to central sensitization in spinal circuits (Schlegel et al., 2004). One possibility is

that peripherally-restricted KOR agonists decreased the response of primary afferents to mechanical stimulation. This decrease in primary afferent activity could be insufficient to activate the sensitized spinal circuits. An alternative or concurrent possibility is that due to the higher likelihood of encountering a noxious thermal stimulus in cutaneous tissues, it could be that more afferents are tuned for noxious heat detection and more easily sensitized to a heat stimulus following injury. Our data suggests that KOR is not expressed on noxious heat-tuned afferents, and hence cutaneous KOR-expressing afferents may be in the minority. Even if KOR agonists led to these fibers releasing less CGRP and SP, it may not be enough to prevent sensitization of non-KOR expressing, noxious heat-tuned afferents (many of which also express and release CGRP and SP).

4.3 POSSIBLE IMPLICATIONS OF HIGHER TRANSCRIPT LEVELS IN KOR-EXPRESSING AFFERENTS

An interesting finding of the studies presented here is a deeper characterization of the primary afferents expressing KOR. Using IHC and single-cell RT-PCR, we made the observation that the subgroup of peptidergic afferents expressing KOR are a unique subset, expressing higher transcript levels of the genes encoding for the neuropeptides CGRP and SP, as well as for the receptors TRPV1, TrkA, GFRα3, and PGI_2R. Higher mRNA levels don't necessarily always translate to higher protein expression, but higher levels of these transcripts could possibly indicate that these proteins are playing a prominent role in the function of KOR-expressing afferents.

Previous reviews based on the use of KOR agonists in preclinical models have summarized the effectiveness of opioid agonists, particularly KOR agonists, in reducing pain in inflammatory states (Millan, 1990); (Vadivelu et al., 2011). Several factors may contribute to this effect, such as increased synthesis and trafficking of these receptors to peripheral afferent terminals, larger effects of opioid receptors due to increase of cAMP levels in afferents during inflammation, and breakdown of the perineurium that allows for opioid peptide penetration to sensory afferents (for review, see (Stein & Zollner, 2009); (Vadivelu et al., 2011)). KOR-mediated inhibition of VGCC in a subgroup of primary afferents that likely express high levels of neuropeptides CGRP and SP, key contributors to neurogenic inflammation, would likely decrease the magnitude of neuropeptide release at peripheral terminals and thereby decrease the feedback loop in the periphery that is at the core of neurogenic inflammation, present in both inflammatory pain and itch states (Grant et al., 2002); (Rosa & Fantozzi, 2013).

Growth factors and their receptors play a critical role in the development of primary afferents early in development, but later in development shift to regulating sensitization of afferents, which is common in inflammatory states and after injury (Zhu & Oxford, 2011); (Jankowski & Koerber, 2010). KOR-expressing afferents display high transcript levels for growth factor receptors TrkA (receptor for nerve growth factor) and GFRα3 (receptor for artemin). NGF and artemin have been shown to both sensitize afferents and behavioral responses (for review, see (Jankowski & Koerber, 2010)) and also have been shown to potentiate TRPV1 responses (Ikeda-Miyagawa et al., 2015); (S. A. Malin et al., 2006) – *Trpv1* being a transcript that was also significantly elevated in KOR-expressing neurons. Thus, KOR-mediated inhibition of these neurons in particular could be a contributing factor as to why KOR agonists are particularly effective in reducing pain in inflammatory states. Future studies could investigate

KOR-expressing afferents innervating different tissue types and how their responses are changed by different growth factors. Even though our results show a similar pattern of *Grfα3* and *Trka* transcript expression in *KOR-cre* saphenous and femoral afferents, previous work has shown that muscle and cutaneous afferents show different levels of sensitization to artemin and NGF (S. Malin et al., 2011). It would be interesting to compare sensitization of KOR-expressing muscular and cutaneous afferents to different growth factors. This type of experiment could help to test whether the similar transcriptional profile of KOR-expressing afferents indicates these afferents are involved in detecting like stimuli across tissue types.

Another contributing factor to the effectiveness of KOR agonists in inflammatory states is higher expression of *Ptgir*, the transcript for the prostacyclin (PGI_2) receptor. Similar to PGE_2, PGI_2 is also involved in generation of inflammatory states and pain sensitivity. PGI_2R knockout mice demonstrate decreased vascular permeability and paw volume following injection of algogens bradykinin or carrageenan, respectively. These knockout mice showed decreased nociceptive responses in the acetic acid writhing test suggesting a role for this receptor in inflammation and pain generation (Murata et al., 1997). As the PGI_2 receptor is expressed on peripheral tissues, such as blood vessels (Myren, Olesen, & Gupta, 2011), as well as primary afferents (Oida et al., 1995), it is like the cellular mechanisms underlying the effect prostacyclin and its receptor during inflammatory pain is complex and involves multiple tissue types. However, KOR-mediated inhibition of primary afferents expressing this receptor would likely play a part in decreasing sensitization but also decreasing activation following inflammation or injury, as PGI_2 receptor activation has been shown to increase adenylyl cyclase activity and cAMP production (Nakae et al., 2005). Targeting primary afferents expressing the PGI_2 receptor,

therefore, may be helpful in reducing ongoing pain in addition to evoked pain following an injury.

4.4 A POTENTIAL ROLE FOR KAPPA OPIOIDS IN THE REGULATION OF LOW-THRESHOLD MECHANOSENSATION

One of the more surprising and novel findings of the experiments described here is the expression of KOR in several subtypes of LTMRs. We found that some NF200-positive lanceolate and circumferential endings targeting hair follicles in the hind paw and back skin express KOR. Circumferential endings have been identified and described as two subtypes, those expressing NF200 and those expressing CGRP (Abraira & Ginty, 2013); (Bardoni et al., 2014). Bai et al. (Bai et al., 2015) used a genetic mouse model to identify and characterize the NF200-expressing subset of primary afferents that form circumferential endings. The authors show that these afferents respond preferentially to skin stroking but are unresponsive to hair deflection, and encode skin deflection into the noxious range. In contrast, lanceolate endings respond preferentially to hair deflection and non-noxious skin indentation (Bai et al., 2015); (Rutlin et al., 2014). NF200-expressing circumferential endings are also among the most expansive in terms of the region of skin innervated by a single afferent, and can up to 180 circumferential endings in the skin, up to $3mm^2$ area of skin (Bai et al., 2015). NF200-expressing afferents forming lanceolate endings innervate fewer hair follicles and a smaller area of skin (Bai et al., 2015). This suggests that, even if only a small number of these subtypes of LTMRs express KOR, these afferents could innervate a relatively large area of skin and number of hair follicles. Therefore,

KOR signaling could have a larger effect on the transmission of low-threshold mechanosensation in the hairy skin than the number of LTMR afferents would suggest.

The next question, of course, is what effect KOR signaling would have on LTMRs and the implications of this signaling for mechanosensation. One possibility is that the kappa opioid system is regulating the overall course-scale sensitivity of the skin to stimulation opposed to fine-scale modulation of an individual aspect of low-threshold mechanosensation. We show that KOR agonist dynorphin decreases evoked-EPSCs in lamnia III after optogenetic activation from KOR-expressing primary afferents, and lamina III is part of the LTMR-recipient zone (Abraira et al., 2017). Further studies are needed to definitively show that KOR agonists decrease neurotransmitter release from LTMRs (discussed below in Section 4.5). However, dynorphin signaling in the spinal cord could act as a gain control mechanism for low-threshold mechanosensation. Dynorphin could modulate the sensitivity of LTMR inputs into the spinal cord depending upon the context of incoming signals such that the organism can extract the relevant information in given environmental and physiological contexts. This type of modulation has been studied in multiple sensory systems (Nikolaev, Leung, Odermatt, & Lagnado, 2013); (Robinson & McAlpine, 2009) and several recent papers have investigated this type of gain control modulation by dopamine in the olfactory bulb (Banerjee et al., 2015); (Vaaga, Yorgason, Williams, & Westbrook, 2017).

One context in which a potential gain control role of kappa opioid signaling on low-threshold mechanosensation would be useful is mechanical allodynia. There is a previous report in the literature characterizing DOR expression in LTMR subgroups which led to the speculation that DOR agonists may be effective in the treatment of mechanical allodynia (Bardoni et al., 2014). Together these data suggest a conceivable role of the opioid receptor system in the

regulation of mechanical sensitivity. One possibility is that after injury, opioid signaling in the dorsal horn is elevated in an effort to decrease excitability such that the pain sensation is still felt (to protect from further tissue damage), but not to the extent that the animal cannot function (in case it still needs to run away from a predator). However, it seems that this type of modulation is a delicate balance, as elevated dynorphin levels in the spinal cord following injury can also exacerbate sensitivity (Laughlin et al., 1997); (Koetzner, Hua, Lai, Porreca, & Yaksh, 2004).

4.5 THE ROLE PERIPHERAL KOR SIGNALING HAS ON MODULATION OF ITCH AND PAIN

We found that optogenetic activation of *KOR-cre* afferents caused behaviors consistent with nocifensive withdrawal, but did not elicit scratching behaviors, and that KOR agonists decreased capsaicin-induced licking of the hind paw. These data seems in contrast with a previous report from our laboratory that included an experiment testing the effect of systemically administered nalfurafine on itch- and pain-related behaviors (Kardon et al., 2014) using the cheek model (Shimada & LaMotte, 2008). In this experiment, either the pruritogen chloroquine or the algogen capsaicin was injected intradermally into the cheek and the number of scratch bouts (indicative of itch-related behavior) or wipes (indicative of pain-related behavior) was quantified. The results showed that nalfurafine significantly decreased the number of scratch bouts caused by both chloroquine and capsaicin, but had no effect on the number of wipes induced by either chemical. This was interpreted as KOR agonists having a specific effect on itch, but not pain. In contrast, the experiments described here demonstrate that systemically administered nalfurafine, as well as peripherally-restricted ICI204,488 and FE200665, decreased the time spent licking the

hind paw following intraplantar injection of capsaicin which is interpreted as KOR agonist-mediated decrease in pain-related behaviors.

The question is why nalfurafine would decrease pain behaviors in one assay but not in the other, and there are several possible explanations. The first possibility is the location of the capsaicin injection, one being intradermal and primarily affecting cutaneous fibers, and the other being intraplantar which could affect cutaneous afferents but also afferents targeting deeper tissues, such as the muscle. It could be that KOR-expressing fibers targeting the skin are more tuned for pruritogens than for algogens, and KOR-expressing fibers targeting deeper tissues are more tuned for algogens. Although the *KOR-cre* neurons backlabeled from the saphenous nerve showed a similar pattern of transcripts to those backlabeled from the muscle, it could be that the cutaneous KOR-expressing fibers also express other proteins that would tune them for pruritoception, such as the chloroquine receptor Mrgpra3. However, this is not what was observed in the experiments described here, as only one of the *KOR-cre* neurons expressed MrgprA3 mRNA. The sample size in this experiment was relatively small, though (n=9 cells), and so this doesn't exclude the possibility that some cutaneous KOR-expressing cells also express Mrgpra3. Nevertheless, the data collected thus does not suggest that cutaneous KOR-expressing fibers are specific for itch but other experiments would be necessary to arrive at a more definitive conclusion. Further, nalfurafine would be acting at both peripheral and central KORs, not only at peripheral KORs. Other experiments described here, in addition to others (Y. Wang et al., 2005); (Inan & Cowan, 2004); (Suzuki et al., 2004); (Endoh et al., 2001), demonstrate that nalfurafine decreases itch- as well as pain-related behaviors and therefore is consistent with the interpretation that KOR agonists do not specifically modulate itch sensation.

An alternative and / or contributing factor could be that itch is under strong modulatory control, especially within dorsal horn circuits. Loss of B5-I neurons in *bhlhb5* mutant mice caused exacerbated itch such that mice developed lesions from excessive scratching and grooming (S. E. Ross et al., 2010). One of the two subsets of B5-I neurons are inhibitory interneurons that also express the endogenous KOR agonist dynorphin (Kardon et al., 2014). This suggests the possibility that loss of one component of modulatory controls in the spinal cord regulating the processing of itch sensation has dramatic consequences. Therefore, any modulation of this control by exogenous KOR agonists would have a stronger and perhaps more noticeable impact on itch-related behaviors than pain-related behaviors.

It could also be that intraplantar injection into the hind paw causes itch and/or pain sensation, but that mice cannot scratch their hind paw so we interpret the behavior as licking, a nocifensive behavior. In the present experiment, we did not attempt to distinguish between licking and biting of the hind paw, which could be an indication of itch compared to pain and is used this way in the calf model (Shimada & LaMotte, 2008). Another possibility is that there could have been some methodological inconsistencies in the previous experiment reported in the Kardon et al. (2014) paper. The behavior that is considered wiping in response to the injected chemical is very specifically described in the original report of the model (Shimada & LaMotte, 2008). If one was not very careful and observant while scoring the experiment, grooming behavior and/or a scratching-like of behavior with the forelimb could be mistakenly counted as wiping behavior, thus leading to an overestimation of the number of wipes caused by a chemical and masking an effect (if there was one). One or all of these alternatives could contribute to the differences observed between Kardon et al. (2014) and the experiments reported here.

4.6 FUTURE DIRECTIONS

4.6.1 Developmental KOR expression

Using both a cre-dependent allele and a cre-dependent virus in combination with the *KOR-cre* allele, we could carefully characterize the cell types expressing KOR throughout development. If KOR-expressing afferents were labeled from embryonic development through adulthood, approximately one third of the lumbar DRG afferents in the adult showed *KOR-cre* labeling, and a similar proportion of the DRG afferents in the adult showed *KOR-cre* labeling if afferents were labeled early in postnatal development (at P1). However, if KOR-expressing afferents were labeled during adulthood, a smaller proportion of the total lumbar DRG afferents showed labeling such that it was similar to the 9% of DRG neurons expressing KOR that was previously reported by Ji et al. (1995). While cre-dependent viral labeling approaches are not 100% efficient, the dual FISH experiments showed that it is possible to label upwards of 90% of *Oprk1*-expressing afferents using this approach. Together these data suggest that KOR is more widely expressed during early stages of development up to at least the early postnatal days, and then expression is decreased such that fewer afferents express KOR in the healthy adult mouse.

The downregulation of KOR expression in DRG afferents is not completely unexpected, as many genes show different patterns of expression throughout development. In the DRG, for example, TRPV1 is more widely expressed early in development such that both peptidergic and non-peptidergic C-fibers express TRPV1 early and this expression is narrowed to a subset of peptidergic fibers in adulthood (Cavanaugh et al., 2011). Previous studies investigating MOR and DOR immunoreactivity in the rat DRG across development also observed a decrease in the percentage expressing these proteins by approximately 10% from P3 to P21. This decrease was

most notable in NF200-expressing cells and in adulthood almost all MOR and DOR-expressing afferents were restricted to small and medium diameter neurons (Beland & Fitzgerald, 2001); (Nandi et al., 2004). Together, this study and the results of our experiments support the idea that the opioid system plays different roles in modulating somatosensory information in neonatal development versus adulthood. Consistent with this idea, Nandi et al. (2004) investigated the effects of MOR agonists on analgesia in neonate versus adult animals and report that the potency of morphine is higher in neonates than adults, particularly for mechanical stimulation.

Some evidence suggests that KOR agonists may have a similar effect; Barr et al. (Barr et al., 2003) report that an intraplantar injection of KOR agonist U50,488 reduced nocifensive behavior in the formalin test in rats at P3 and P21. Further studies would be necessary to confirm this effect using different agonists, as U50,488 also has some affinity for voltage-gated sodium channels (Joshi et al., 2003). Yet, using peripherally-restricted KOR agonists instead of centrally-penetrant drugs such as morphine in the treatment of pain for infants is appealing because they likely would have fewer side effects. Peripherally-restricted KOR agonists could also have the benefit of a smaller impact on the development of central circuits. A recent report found that repeated morphine injections in rats decreased the potency of the anesthetic lidocaine, which is used in surgical procedures (Liu & Gold, 2016). Whether a similar effect on lidocaine potency is observed when using a KOR agonist would be an interesting next step, and extending this type of study to include neonates and adults could better increase the understanding of post-surgical pain treatment.

4.6.2 KOR expression in primary afferents after injury

Our study focused on characterizing KOR-expressing primary afferents in naive adult mice and found that at least two distinct groups of primary afferents express KOR. However, we did not investigate how KOR expression in primary afferents changes after injury. Several studies have reported increases in *Oprk1* mRNA expression or KOR protein expression in a model of neuropathic pain (Caram-Salas et al., 2007), after PGE2-induced inflammation (Zambelli et al., 2014), or after CFA-induced inflammation (Puehler et al., 2006); (Obara et al., 2009). Now that the subset of primary afferents expressing KOR have been characterized in more detail, it would be interesting to determine if the increase in KOR expression after injury is due to an upregulation in the same afferents expressing KOR in a naïve state, if KOR expression is induced in a different subset(s) of primary afferents, or both. Because we saw a decrease in the proportion of afferents expressing KOR from early development to adulthood, it would be interesting to test whether or not primary afferents that expressed KOR at one point in development are more likely to induce KOR expression after an injury.

In the studies described here, we did not observe overlap of KOR with TRPM8 and therefore did not test the effects of KOR agonists on cold sensitivity. Interestingly, though, a recent study from the McKemy lab found that GFRα3 KO mice showed selective reductions in cold allodynia in models of inflammatory, neuropathic, and chemotherapy-induced pain (CPN) (Lippoldt, Ongun, Kusaka, & McKemy, 2016). This lab has previously described artemin, GFRα3 ligand, induces sensitization to cold stimuli in a TRPM8-dependent manner (Lippoldt, Elmes, McCoy, Knowlton, & McKemy, 2013). We did not observe overlap of *KOR-cre* and TRPM8-IR, nor did any of the *Oprk1* positive neurons express *Trpm8* mRNA. One would

predict that peripherally-restricted KOR agonists would not affect cold pain or cold hypersensitivity, but it could be that neurons expressing GFRa3 and TRPM8 could express KOR after injury and therefore KOR agonists may alter processing of cold in an injured state. The effect of KOR agonists on cold responsive primary afferents in naïve states and after tissue injury, as well as behavioral responses, remains to be tested.

4.6.3 Testing the effects of dorsal horn presynaptic KORs in response to natural stimuli or in specific neuronal subtypes

In this study, we found that KOR agonist dynorphin decreased light-evoked EPSCs from *KOR-cre* primary afferent terminals in neurons in both lamina I and lamina III or the dorsal horn. Terminals in these laminae are also more likely to be functionally distinct in that afferents targeting lamina I are more likely to transmit nociceptive information and those in deeper lamina (II-V) are more likely to transmit low-threshold mechanosensation. However, this conclusion cannot be definitely reached without more specific experiments. Future studies using the *ex vivo* skin-nerve-spinal cord preparation or in vivo recordings targeting lamina I neurons could determine the effect of KOR agonists on presynaptic release caused by natural stimulation of the skin. Considering the results of the behavioral assays, it would be interesting to compare the effect of spinally administered KOR agonists on presynaptic release when using chemical algogens, chemical pruritogens, or noxious thermal stimuli. If KOR agonists don't have an effect on presynaptic release caused by thermal stimuli, it could be that thermal sensitivity is being modulated by the kappa opioid system at a level other than the primary afferents. This separation between the effect of peripheral and central kappa system in regulating a particular modality of nociception would be an interesting concept to explore in the future as the

modulation of somatosensation by the opioid system throughout the pain pathway continues to be explored.

As it is would be difficult to access dorsal horn neurons in lamina III in a whole spinal cord recording, a possible way to more specifically test the effect of KOR agonists on glutamate release from LTMR central terminals is to use the spinal preparation developed by Yu Omori (and described in Chapter 3) in combination with genetic mouse lines. The laboratory of David Ginty has developed several mouse lines that specifically target subtypes of hair follicle afferents. Using optogenetics to activate particular hair follicle types, such as myelinated circumferential afferents (Bai et al., 2015) and either Aβ or Aδ lanceolate endings (L. Li et al., 2011); (Rutlin et al., 2014).

4.6.4 Possible ways to test the effects of KOR agonists on behavior driven by low-threshold mechanosensation

In our studies, we focused on the effect of centrally-penetrant and peripherally-restricted KOR agonists primarily on behaviors elicited by aversive stimuli, such as injection of a noxious chemical or exposure to a noxious heat source. Behavioral studies investigating the low-threshold mechanosensation have mainly been focused on the glabrous skin, such as the von Frey test and dynamic mechanical sensation assay (Duan et al., 2014). However, there are very few hairs on the ventral footpad of the mouse making it difficult to assess the contribution of LTMRs innervating the hairy skin to behavioral responses to begin understanding how the activity of these afferents contribute to the sensation generated. A few tests have been designed to test how low-threshold mechanosensation is altered in mice with genetic mutations. An altered version of the sticky tape assay was used to test the effects that knockout of the

mechanosensitive ion channel *Piezo2* had on mice's perception of the tape stuck to their back (Ranade et al., 2014). The authors found that *Piezo2* knockout mice showed fewer attempts to remove the tape (i.e., less licking and scratching) than wild-type littermates and interpret this as a decrease in sensitivity to low-threshold mechanosensation. However, as the *Piezo2* knockout mice also showed deficits in proprioception, it is unclear if this result was truly a measure of low-threshold mechanosensation or the agility of the mice compared to controls and this particular assay would need to be further validated.

One other model that could be used to test the effects of KOR signaling on low-threshold mechanosensation, originally developed to test sensory integration in a mouse model of autism, is the tactile pre-pulse inhibition (Tactile PPI) assay (Orefice et al., 2016). In this assay, a light puff of air is given as a pre-pulse 'warning' that acts to dampen the startle response to a loud tone. Mice that have been shaved and received lidocaine injection (to block primary afferent activity) do not show this dampening of the startle response, suggesting that the tactile pre-pulse is dependent on the activity of the primary afferents responding to the air puff; these afferents have previously been shown to be those that form lanceolate endings (Bai et al., 2015); (Rutlin et al., 2014). KOR-expressing neurons form lanceolate endings, which would respond to the air puff pre-pulse, but they also form circumferential endings which would not respond to the air puff. Thus, it is unclear if KOR expression on lanceolate endings alone would be strong enough to drive a change in sensitivity to the air puff. Studies using the Tactile PPI experimental paradigm or variations of this assay would be interesting to explore in the future.

While a mouse may not the best model system to investigate the hairy LTMR system, it does offer a way to investigate the cellular and circuit mechanisms that is not afforded by other systems. The best approach may be to test the effect of peripherally-restricted KOR agonists on

low-threshold mechanosensation in humans that can report and describe the sensation they are experiencing, though, and then study possible mechanisms in a mouse model.

4.6.5 Investigating interactions between the kappa opioid system and the immune system

In the studies described here, we characterize the primary afferent nerves that express the KOR, and use exogenous KOR agonists to test the effect of KOR signaling on pain and itch behaviors. We find that peripherally-restricted KOR agonists are sufficient to decrease behavioral responses after injection of chemical algogens or pruritogens, as well as mechanical hypersensitivity after an incision injury. These results are consistent with the pattern of KOR expression on primary afferent nerves, and one highly likely site of action for KOR agonists, especially the peripherally-restricted KOR agonists, is on the peripheral terminals of primary afferent nerve fibers. Another possibility, however, is that the KOR agonists are acting also at KORs expressed on immune cells. Endogenous opioid peptides have been shown to be released by infiltrating immune cells following an injury, including beta-endorphin and dynorphin, that are thought to contribute to endogenous analgesia, at least in part, on opioid receptors on primary afferents (Rittner et al., 2001); (Stein, Hassan, Lehrberger, Giefing, & Yassouridis, 1993); (M. Schafer, Carter, & Stein, 1994); (Cabot, Carter, Schafer, & Stein, 2001). However, there is some evidence that KORs are expressed by certain types of immune cells. Some studies have shown KOR expression on lymphocytes (Gaveriaux et al., 1995); (Chuang et al., 1995) and that activation of these receptors can alter antibody responses (Guan, Townsend, Eisenstein, Adler, & Rogers, 1994); (Gaveriaux-Ruff, Simonin, Filliol, & Kieffer, 2003). Other studies from the Machelska laboratory have shown that approximately one third of leukocytes infiltrating the area of a nerve injury release endogenous opioid peptides (Labuz et al., 2009),. They also report that exogenous

opioid agonists can cause release of endogenous opioid peptides from leucocytes, and that leucocyte depletion decreases the analgesic effects of exogenous opioid agonists in a model of neuropathic pain (Celik et al., 2016). These results suggest that opioid receptors on immune cells could play an important role in the analgesic or antipruritic effects of exogenous opioid receptor agonists.

As several of the behavioral assays used here are likely to induce an inflammatory response, it is possible that KOR on infiltrating immune cells could contribute to the effects of KOR agonists. One possible way to dissociate the contributions of KOR on primary afferents and immune cells would be to knockout KOR in each cell type measure the effects of KOR agonists in behavioral assays of acute itch and pain. To do so, the floxed KOR allele could be used, in which the KOR gene is surrounded by loxP sites, and this mouse could be crossed to a mouse line that expresses cre either in primary afferents or in immune cells. A recent study using a conditional knockout (cKO) of the MOR in Nav1.8-expressing sensory neurons (primarily C-fibers) reported that cKO mice showed reduced opiate-induced analgesia compared to wild-type controls (Weibel et al., 2013). These data suggest that MORs on primary afferents play a critical part in exogenous opioid agonist-induced analgesia. Overall, it is likely that KORs expressed on both the nervous system and the immune system contribute to the decrease in pain or itch behavior observed after KOR agonist treatment. Understanding this interaction and the contribution of KOR signaling within each system to analgesia / antipruritus could help advance treatments. The murine immune system is quite different than the human immune system (Mestas & Hughes, 2004) and so results from these experiments should be interpreted with caution and validated in human tissues if possible.

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