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Investigating Mechanisms in Nociceptors Driving Ongoing Activity and Ongoing Pain

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INVESTIGATING MECHANISMS IN NOCICEPTORS DRIVING ONGOING ACTIVITY AND ONGOING PAIN

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Dedication

To my mother, who has always been an endless source of support and encouragement.

And to Zach, who has been by my side every step of the way and always reassured me that I could accomplish whatever I set my mind to.

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INVESTIGATING MECHANISMS IN NOCICEPTORS DRIVING ONGOING ACTIVITY AND ONGOING PAIN

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Ongoing (apparently spontaneous) pain at rest is a major complaint of patients suffering from many forms of acute and chronic pain, including acute and persistent postsurgical pain. Accumulating evidence suggests ongoing activity in nociceptors is a major driver of ongoing pain. Ongoing activity can be generated in sensory neurons in the absence of sensory generator potentials if one or more of three neurophysiological alterations occur – prolonged depolarization of resting membrane potential (RMP), hyperpolarization of action potential (AP) threshold, and/or increased amplitude of depolarizing spontaneous fluctuations of membrane potential (DSFs) to bridge the gap between RMP and AP threshold. Cellular signaling pathways that increase DSF amplitude and promote ongoing activity acutely in nociceptors were unknown for any neuromodulator, as were the neurophysiological alterations that underly ongoing activity in nociceptors after deep tissue incision. The work presented in this dissertation sought to identify a cellular signaling pathway and injury- and inflammation-related neuromodulator that induces alterations that drive hyperexcitability, such as enhanced DSFs, and thereby potentiates ongoing activity in nociceptors, as well as to determine

the alterations that contribute to nociceptor hyperactivity associated with postsurgical pain.

A combination of whole-cell patch clamp electrophysiology, pharmacology, and high content microscopy was used to define the effects of low concentrations of the injury-related proinflammatory mediator serotonin and the major pathway by which it exerts its effects. This study shows that serotonin enhances DSFs, hyperpolarizes AP threshold, and thereby potentiates OA in isolated rat DRG neurons at a concentration of 100 nM, and serotonin exerts these effects on nonpeptidergic nociceptors via the 5-HT_4 receptor and downstream cAMP signaling via PKA and EPAC. Furthermore, whole-cell patch clamp recordings of primary DRG neurons, analysis of spontaneous pain behavior, and RNA sequencing were used to reveal an unexpected persistence of hyperexcitability in isolated DRG neurons and potentially associated differential gene expression, both of which were found after the behavioral expression of postsurgical pain had resolved. In addition to elucidating cell signaling mechanisms that can contribute to acute ongoing activity in nociceptors, these studies set the stage for future investigations addressing important questions about nociceptor contributions to pain.

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List of frequently used abbreviations

5-HT: serotonin

5-HTR: serotonin receptor

8-Br-cAMP: 8-bromo-cyclic adenosine monophosphate

ANOVA: analysis of variance

AP: action potential

cAMP: cyclic adenosine monophosphate

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

$Ca_V3.2$: voltage-gated calcium channel 3.2

CGRP: calcitonin gene-related peptide;

CI: confidence interval

CSF: cerebrospinal fluid

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DRG: dorsal root ganglion

DSF: depolarizing spontaneous fluctuation

EC_{50}: concentration that produces the half maximal stimulatory effect

ECS: extracellular solution

EPAC: exchange protein activated by cAMP

GPCR: G protein-coupled receptor

HCN: hyperpolarization-activated cyclic nucleotide-gated channel

IB4: isolectin B4

IC_{50}: concentration that produces the half maximal inhibitory effect

ICS: intracellular solution

myr-PKI 14-22: myristoylated protein kinase inhibitor 14-22

NA: nonaccommodating

OA: ongoing activity

PGE_2: prostaglandin E_2

PGP9.5: protein gene product 9.5

PKA: protein kinase A

RA: rapidly accommodating

RMP: resting membrane potential

RNA: ribonucleic acid

SCI: spinal cord injury

TRPV1: transient receptor potential vanilloid 1

TTX-R: tetrodotoxin-resistant

Veh: vehicle

Chapter 1

Introduction

Pain is defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.” Pain, while unpleasant at the least and unbearable at the worst, is integral to survival. Pain tells us to remove ourselves from something or stop engaging in an activity that will cause harm. It is also pain that lets us know to protect ourselves when we are vulnerable while a wound or infection is healing. People with gene mutations that cause congenital insensitivity to pain often accumulate injuries that may go undetected and can ultimately lead to reduced life expectancy (Daneshjou et al., 2012; Mifsud et al., 2019; van den Bosch et al., 2014). Sensitivity to pain can help us live long and healthy lives. However, pain can become pathological; when pain is no longer alerting us to actual or potential injury but becomes persistent, it can exact a heavy toll on an individual and on society. Chronic pain is experienced by at least 11% of adults in the United States and is one of the most common reasons adults seek medical care (Dahlhamer et al., 2018). Ongoing spontaneous pain is often the worst complaint of patients suffering from many forms of pain (Backonja and Stacey, 2004; Bennett, 2012). Importantly, severe and persistent pain can tremendously impair quality of life, and current treatment options are often inadequate. Thus, it is important to better understand the mechanisms that drive pain and how it becomes pathological.

1.1. The physiology and pathophysiology of pain: an overview of the alterations that underly acute and chronic pain

Pain and other sensory information is transduced by primary afferents (sensory neurons). Different classes of sensory neurons are specialized to respond to different

types of stimuli. Low threshold mechanoreceptor neurons (LTMRs) are mostly made up of large myelinated Aβ-fibers and some of the smaller myelinated Aδ-fibers. Many of these fast-conducting neurons respond to innocuous mechanical stimuli, such as light touch. Nociceptors, sensory neurons specialized for the detection of injury and inflammation (Basbaum et al., 2009; Das, 2015; Dubin and Patapoutian, 2010), mostly have unmyelinated, small-diameter C-fibers exhibiting low conduction velocity. These respond to noxious mechanical, heat, cold, and/or chemical stimuli. Many Aδ neurons and a small subset of Aβ neurons are also nociceptors.

Under normal conditions tissue damage activates peripheral terminals of nociceptors. This initiates electrical signals, action potentials (APs), that travel along the axons of the nociceptors to the spinal cord, where they synapse with second-order interneurons in the dorsal horn of the spinal cord. The electrical signal is relayed to second-order neurons by synaptic release of the neurotransmitter glutamate and one or more neuropeptides, including substance P and calcitonin gene-related peptide (CGRP) (D'Mello and Dickenson, 2008; Yam et al., 2018). This signal excites second-order neurons, generating APs that travel along their axons, many of which cross over the spinal cord to the spinothalamic tract, then traveling up to the thalamus of the brain. Thalamic third-order neurons then relay the signal to the region of the somatosensory cortex which maps onto the region of the body where the signal originated, as well as to other brain regions such as the insula and anterior cingulate cortex (Bushnell and Apkarian, 2005). This results in the conscious perception of pain in the injured area. Additionally, there are descending pathways that enhance or suppress synaptic transmission in the ascending pain pathways (Ren and Dubner, 2008), but the focus here will be on the primary sensory neurons that initiate activity in the pain pathways.

Activation of nociceptors, which normally are electrically silent, produces immediate pain and protective reflexive responses. It also produces sensitization of neural and behavioral responses to subsequent noxious and innocuous stimuli. These sensitizing effects are mediated by alterations in pain pathways. One sensitizing effect is enhanced responsiveness of primary sensory neurons to stimulation of their receptive fields (peripheral sensitization). Another is enhanced responsiveness at the level of the spinal cord and brain to sensory input from the periphery (central sensitization) (Meacham et al., 2017). At the peripheral terminals, enhanced sensory generator potentials and reduced AP threshold lead to increases in nociceptor discharge and input to the central nervous system (CNS). If injury or infection is severe enough to damage peripheral nerves, ectopic generation of APs can occur in the axons (often at neuromas) or the cell bodies of sensory neurons (Walters, 2019), which are clustered in the dorsal root ganglia (DRG) or trigeminal ganglia.

Increased nociceptor activity can produce synaptic plasticity (Price and Inyang, 2015; Reichling and Levine, 2009), including long-term synaptic potentiation (LTP) and heterosynaptic facilitation (Klein et al., 2008; Pace et al., 2018), which amplifies signals in the pain pathways and contributes to central sensitization. A reduction in inhibitory tone at the presynaptic terminals of nociceptors may also accompany enhanced activity (Comitato and Bardoni, 2021; Gradwell et al., 2020; Guo and Hu, 2014; Hughes and Todd, 2020). In the brain, cortical remodeling can contribute to augmented pain perception (Puntillo et al., 2021). Furthermore, increased facilitation and decreased inhibition by descending modulatory pathways can further enhance transmission of nociceptive information at the level of the spinal cord (Ossipov et al., 2014).

Reduced threshold and subsequent enhanced responsiveness of nociceptors to stimulation underlies hyperalgesia, an increased sensation of pain from a stimulus that normally provokes pain. In addition, central sensitization allows for activity in LTMRs to activate pain pathways, resulting in allodynia, sensation of pain in response to normally innocuous stimuli. In addition to hyperalgesia and allodynia, ongoing (apparently spontaneous) pain at rest is a major complaint of patients suffering from many forms of acute and chronic pain. Accumulating evidence links ongoing pain with ongoing activity (OA, also known as spontaneous activity) that may occur in sensory neurons and their downstream targets in the spinal dorsal horn in the absence of an apparent stimulus. The findings that activation of even a few human nociceptors *in vivo* evokes immediate pain (Nagi et al., 2019; Ochoa and Torebjörk, 1989) and the high incidence of OA in C-fiber nociceptors in microneurographic recordings from patients with ongoing neuropathic pain (Kleggetveit et al., 2012; Serra et al., 2012) indicate that a major driver of ongoing human pain is OA in nociceptors.

1.2. Observing drivers of pain in isolated rodent DRG neurons

The OA observed *in vivo*, can be retained in nociceptor somata dissociated from excised DRG. Electrophysiological recordings from single DRG neurons dissociated from human patients (North et al., 2019) and recordings from rodent nociceptors in preclinical neuropathic, inflammatory, and incisional pain models (Bedi et al., 2010; Djouhri et al., 2006; North et al., 2019; Xu and Brennan, 2010) demonstrate an increased incidence of OA in DRG neurons linked to ongoing pain. The occurrence of pain-related OA in dissociated nociceptors in vitro that is similar in pattern and incidence to that occurring in vivo during persistent neuropathic pain (Bedi et al., 2010) provides a special

opportunity to define the cellular mechanisms in nociceptors that enable their ongoing and spontaneous activity.

Examination of OA in isolated DRG neurons has allowed us to make great strides in our understanding of the electrophysiological alterations that drive a sensory neuron from a silent state to a state of OA. As illustrated by our findings in a model of neuropathic pain (Odem et al., 2018), three basic neurophysiological alterations can generate OA in nociceptors in the absence of sensory generator potentials – prolonged depolarization of the resting membrane potential (RMP), hyperpolarization of the action potential (AP) threshold, and an increase in the incidence of large depolarizing spontaneous fluctuations (DSFs) of membrane potential that can intermittently bridge the gap between RMP and AP threshold (Figure 1). While alterations in RMP and AP threshold are commonly recognized contributors to neuronal excitability, the importance of DSFs has only recently been recognized. Large DSFs are prominent in neurons with OA, and they play a major role in controlling the low firing rate and irregular pattern of OA observed in DRG neurons dissociated in animal models of persistent pain, including chronic constriction injury of the sciatic nerve (Study and Kral, 1996), spinal cord injury (Berkey et al., 2020; Odem et al., 2018), and treatment with the chemotherapeutic cisplatin (Laumet et al., 2020), as well as in DRG neurons taken from humans with neuropathic pain (North et al., 2019).

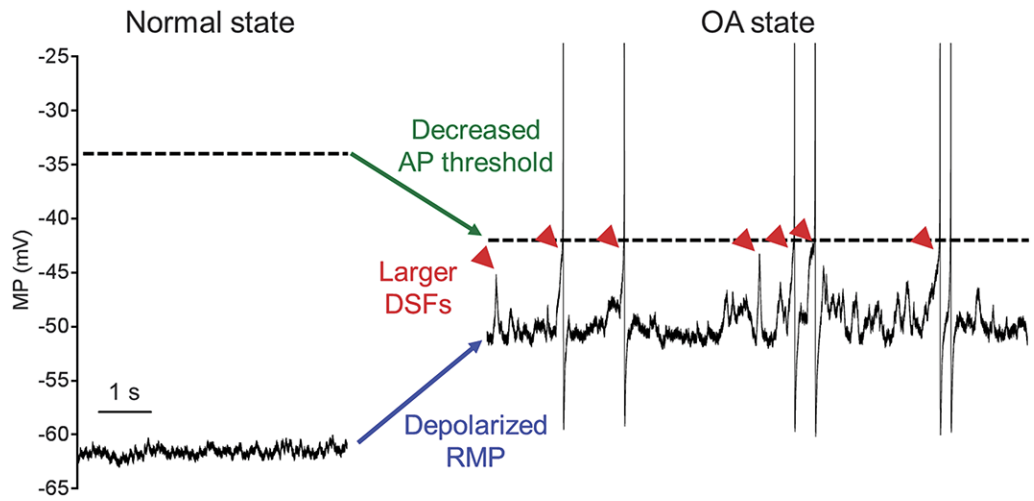


Figure 1. Summary of neurophysiological specializations that promote OA in nociceptors. Representative traces from naïve rats (normal state) and rats with spinal cord injury (OA state). AP, action potential; DSF, depolarizing spontaneous fluctuation; MP, membrane potential; OA, ongoing activity; RMP, resting membrane potential. Reprinted from Title: Isolated nociceptors reveal multiple specializations for generating irregular ongoing activity associated with ongoing pain; Authors: Max A. Odem, Alexis G. Bavencoffe, Ryan M. Cassidy, Elia R. Lopez, Jinbin Tian, Carmen W. Dessauer, and Edgar T. Walters; Publication: *Pain*; Publisher: Wolters Kluwer Health, Inc.; Date: July 12, 2018. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Wolters Kluwer Health, Inc.

As described above, DRG neurons are a diverse population of sensory neurons that are specialized for different sensory modalities. Cutaneous LTMRs sense non-noxious mechanical force; cold receptors respond to cold; and overlapping subsets of nociceptors respond to noxious mechanical force, noxious heat, and noxious chemicals. DRG neurons can be classified based on a variety of molecular markers, cellular properties, and gene expression profiles. LTMRs are relatively large in size compared to nociceptors and express Tropomyosin receptor kinase B (TrkB, a receptor for brain-derived neurotrophic factor [BDNF]) or TrkC (a receptor for neurotrophin-3 [NT-3]). Cold

receptors have small somata, express transient receptor potential melastatin 8 (TRPM8), and do not express CGRP or bind isolectin B4 (IB4) (Dhaka et al., 2008; Peier et al., 2002; Takashima et al., 2007). Nociceptors are a diverse group with mostly small to medium soma sizes. One common way to classify nociceptors is by expression of CGRP (marking “peptidergic” nociceptors) or binding of IB4 (marking “non-peptidergic” nociceptors), as CGRP and IB4 binding distinguish largely separate populations in mice with only a relatively small subset of nociceptors positive for both. A larger overlap between CGRP-expressing and IB4-binding DRG neurons is observed in rats (Price and Flores, 2007). In mice, all peptidergic nociceptors express transient receptor potential vanilloid 1 (TRPV1), while a subset of non-peptidergic nociceptors express this channel, which is well known for its involvement in detection of heat and pain, especially in response to capsaicin, the component of chili peppers that causes a burning sensation. Recent findings in human DRG neurons show substantial differences from mice in the expression of these nociceptive markers (Shiers et al., 2020).

1.3. Injury- and inflammatory-related signals sensitize neurons

While it is usually nociceptive neurons that transduce the signals that lead to the sensation of pain, the transduction, transmission, and perception of pain are modified and often initiated by modulators released from non-neuronal cells. The peripheral terminals of nociceptors are packed with receptors and ion channels that detect molecular mediators released from immune cells and other cell types during injury and inflammation. During neuroinflammation, glial cells are activated and release pro-inflammatory cytokines and chemokines that contribute to peripheral and central sensitization. Neurons themselves can also release neuropeptides and other signals that influence inflammatory cells, leading to neurogenic inflammation.

1.3.1. Neuroimmune interactions

In response to injury, immune cells release a variety of inflammatory mediators that activate and/or sensitize nociceptors (Figure 2). Mast cells are tissue-resident immune cells found near externally exposed surfaces that act as “first responders” to foreign invaders and tissue damage. Mast cells are known to associate closely with nociceptors and to contribute to the pathobiology of several types of pain, including chemotherapy-induced peripheral neuropathy (CIPN), pelvic pain, and osteoarthritis (Azimi et al., 2016; Breser et al., 2017; Forsythe and Bienenstock, 2012; Gupta and Harvima, 2018; Jaggi et al., 2017; Sousa-Valente et al., 2018). Upon activation, mast cells immediately release preformed granules containing nociceptor-sensitizing mediators, including serotonin (which is of particular interest and will be introduced in detail in Chapter 3), histamine, cytokines (e.g. Interleukin 5 [IL-5], tumor necrosis factor alpha [TNF-α], IL-1β, IL-6, and IL-17), and tryptase. Later, via de novo synthesis, mast cells release cytokines, growth factors (e.g. nerve growth factor), and neuropeptides (e.g. substance P). Meanwhile, macrophages and neutrophils are activated at and mobilized to the site of injury where they also release a host of inflammatory cytokines, growth factors, and lipids (e.g., IL-1β, IL-6, and prostaglandin E_2 [PGE_2]) that can activate and sensitize nociceptors to increase pain. It was demonstrated recently that angiotensin receptor 2-expressing macrophages at the site of nerve injury are a critical component in triggering neuropathic pain after spared nerve injury in mice (Shepherd et al., 2018).

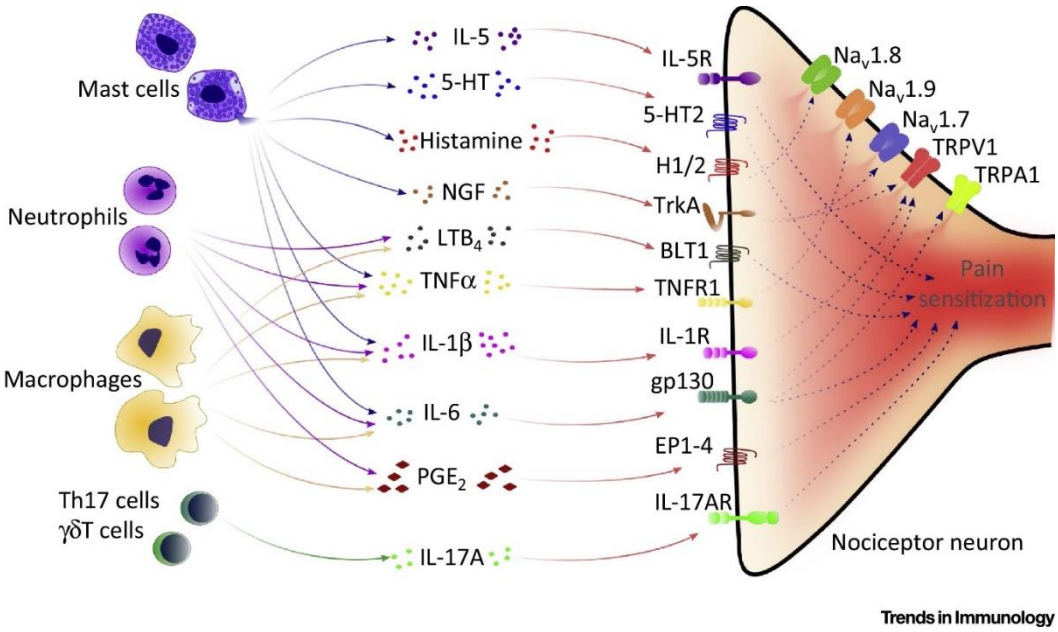


Figure 2. Immune cells release mediators that produce peripheral sensitization of nociceptor sensory neurons and pain. During inflammation, tissue-resident and recruited immune cells secrete molecular mediators that act on the peripheral nerve terminals of nociceptor neurons to produce pain sensitization. In these neurons, specific cytokine, lipid, and growth factor receptor intracellular signaling pathways lead to phosphorylation and/or gating of ion channels $Na_v1.7$, $Na_v1.8$, $Na_v1.9$, TRPV1, and TRPA1, leading to increased action potential generation and pain sensitivity. Reprinted with permission under license. Title: Nociceptor Sensory Neuron–Immune Interactions in Pain and Inflammation; Authors: Felipe A. Pinho-Ribeiro, Waldiceu A. Verri, and Isaac M. Chiu; Publication: *Trends in Immunology*; Publisher: Elsevier; Date: January 1, 2017. License number 5032131331454.

In addition to their roles in nociceptor activation and sensitization at sites of injury, neuroimmune interactions are also known to take place in the DRG where immune cells interact with nociceptor somata to modulate pain. Mast cell activation in the DRG contributes to pain in rodent models of sickle cell disease, sciatic nerve injury, and CIPN (Gao et al., 2016; Saleem et al., 2019; Vincent et al., 2013). Macrophages, monocytes

and neutrophils increase in the DRG in rodent models of radiculopathy, systemic inflammation, CIPN and peripheral nerve injury (Kim and Moalem-Taylor, 2011; Liu et al., 2014; Yoon et al., 2012; Zhang et al., 2016, 2021), and expansion and proliferation of the macrophage populations near the somata of injured sensory neurons makes a critical contribution to the initiation and maintenance of neuropathic pain in a mouse spared nerve injury model (Yu et al., 2020).

Furthermore, mast cells, macrophages, neutrophils, and T cells express receptors for neurotransmitters and neuropeptides, such as vasoactive intestinal peptide, CGRP, and substance P, released from nociceptors during neurogenic inflammation and thus can respond directly to nociceptor activity. Neuropeptides potentiate activation of mast cells (Aich et al., 2015; Kim et al., 2014; Rosa and Fantozzi, 2013). Thus, a feedback mechanism in which mast cell activation in response to tissue damage leads to excitation of nociceptors, which in turn release neuropeptides that cause further activation of mast cells can sustain activity in pain pathways. This cycle of mast cell activation and peripheral nerve sensitization may play a role in the development and maintenance of chronic pain (Austin and Moalem-Taylor, 2010). Similarly, substance P induces the release of pro-inflammatory cytokines from macrophages and monocytes (Lotz et al., 1988; Sun et al., 2008).

1.3.2. Neuroglial interactions

In the move away from a neurocentric view of pain, another cell type that has come into focus as intimately coupled to neurons in pain sensitization is glial cells. Neuroglia include Schwann cells and satellite glial cells (SGCs) in the peripheral

nervous system (PNS) and microglia, astrocytes, and oligodendrocytes in the CNS. The focus here is on glial effects on nociceptors.

Schwann cells interact with sensory nerve fibers and under physiological conditions maintain the local environment, help form the myelin sheath around myelinated axons, and envelop bundles of unmyelinated C-fibers (Kidd et al., 2013). However, nerve injury triggers a phenotypic switch in Schwann cells, causing them to proliferate, migrate, and secrete factors that cause nerve degeneration, regeneration, and inflammation (Jessen and Mirsky, 2019). Secreted factors include the pro-inflammatory mediators TNF-α, IL-1β, IL-6, and PGE_2 (Bolin et al., 1995; Muja and DeVries, 2004; Murwani et al., 1996; Shamash et al., 2002).

Satellite glial cells (SGCs) envelop neuronal somata in the DRG. SGCs couple to each other via gap junctions, and there is evidence suggesting they can synchronize the activity of sensory neurons (Spray et al., 2019). Noxious stimulation or injury of sensory neurons leads to increased gap junction coupling between SGCs and their release of proinflammatory mediators. This leads to increased activity of the sensory neurons and promotes infiltration of immune cells. For example, cisplatin effects on SGCs lead to increased release of TNF-α and IL-6, leading to increased excitability of sensory neurons (Leo et al., 2021).

Sensitized sensory neurons also release colony stimulating factor 1 (CSF1) in the spinal cord, which induces proliferation and activation of microglia (Guan et al., 2016), a specialized population of macrophages that function as glial cells in the CNS. Activated microglia in turn elicit activation and proliferation of astrocytes. These

activated CNS neuroglia contribute to neuroinflammation that can in turn contribute to central sensitization by impacting spinal circuits (Old et al., 2015).

1.4. cAMP signaling in nociceptors

Cyclic adenosine monophosphate (cAMP) is a major mediator of intracellular signal transduction in response to a number of hormones, neurotransmitters, and inflammatory mediators that bind G protein-coupled receptors (GPCRs). GPCRs are a large family of receptors that use G proteins to transduce signals to their effectors, which include enzymes such as adenylyl cyclase (AC) and phospholipase C (PLC) as well as Ca^{2+} channels (Ross and Kenakin, 2001). The G proteins are comprised of two subunits – the α subunit and the βγ subunit. The major effector type and whether the activity of the effector is increased or decreased depends on the α subunit. $G\alpha_s$ stimulates AC activity, while $G\alpha_i$ inhibits AC activity; and $G\alpha_q$ stimulates PLC activity, leading to release of intracellular Ca^{2+} stores and activation of protein kinase C (PKC). Additionally, the Gβγ subunits regulate K^+ channels, AC, PLC, and phosphatidyl inositol-3-kinase (PI3K). Ligands for GPCRs in sensory neurons include an array of biogenic amines (e.g. serotonin and histamine), eicosanoids (e.g. prostaglandins), lipids (e.g. cannabinoids), and peptides (e.g. opioids and vasoactive intestinal peptide). As a result of their abundance and physiological importance, GPCRs are common targets for therapeutics (Ross and Kenakin, 2001).

Several inflammatory mediators, including serotonin, activate GPCRs coupled to $G\alpha_s$, which in turn activate AC, leading to pain sensitization acutely (Villarreal et al., 2009). Recent studies also implicate signaling downstream of AC in the maintenance of chronic pain (Bavencoffe et al., 2016; Dessauer et al., 2017; Sadana and Dessauer,

2009; Vadakkan et al., 2006). AC synthesizes the second messenger cAMP from adenosine triphosphate (ATP). Cyclic AMP can then go on to activate its effectors – protein kinase A (PKA), exchange protein activated by cAMP (EPAC), hyperpolarization-activated cyclic nucleotide-gated channels (HCN), and popeye domain containing proteins (POPDC).

The most extensively studied cAMP effector, PKA, plays an important role in pain sensitization and nociceptor ongoing activity by phosphorylating a multitude of targets, and can exact longer-term changes by modulating gene expression via the transcription factor cAMP-response element binding protein (CREB). PKA-activating inflammatory mediators or direct activation of PKA increases activity of channels involved in nociceptor hyperexcitability, including TRPV1, T-type Ca^{2+} channels, and tetrodotoxin-resistant voltage-gated Na^{+} channels (Kim et al., 2006; Ohta et al., 2006; Vijayaragavan et al., 2004). PKA activation can also increase excitability by negatively regulating K^{+} channels that normally limit excitability and repetitive firing (Nuwer et al., 2010). Furthermore, inhibiting PKA activity or disrupting interaction with its AKAP scaffold in dissociated DRG neurons reduces nociceptor OA in a spinal cord injury model of neuropathic pain (Bavencoffe et al., 2016).

Recently, EPAC has also come to light as an important player with a role in the transition from acute to chronic pain. EPACs are guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP for the Ras-like GTPases Rap1/2, thereby activating them. Downstream of EPAC-Rap, PLC and PLD mediate the translocation and activation of protein kinase C ε (PKCε) (Schmidt et al., 2001), which contributes to sensitization in neuropathic and chronic inflammatory pain (Dina et al., 2001, 2000; Joseph and Levine, 2003a, 2003b; Khasar et al., 1999; Numazaki et al.,

2002; Sweitzer et al., 2004). Thus, EPAC mediates a connection between cAMP and PKC signaling (Hucho et al., 2005), which were previously thought to function as two separate pathways. It is thought that a transition to increased downstream cAMP signaling via EPAC-PKC is involved in nociceptor priming, which is likely a key factor in the transition from acute inflammatory pain to chronic pain (Eijkelkamp et al., 2010; Hucho et al., 2005; Vasko et al., 2014; Wang et al., 2018). In addition to nociceptor priming and persistent pain, there is evidence for the involvement of EPAC in mediating acute inflammatory pain (Goode and Molliver, 2021).

HCN channels represent another cAMP effector that is widely expressed in the PNS and plays a role in neuronal excitability and pain transduction. As the name suggests, these channels are activated by hyperpolarization and modulated by cAMP. In response to hyperpolarization, they conduct Na^+ ions into the cell (and K^+ ions out of the cell), resulting in net depolarization, which causes the channel to close again. Thus, HCN channels have the capacity to act as pacemakers, a function typically associated with HCN in the heart. Furthermore, studies suggest HCN channels in nociceptors are critical players in inflammatory and neuropathic pain (Emery et al., 2011; Ma et al., 2021).

1.5. Conclusions

Ongoing spontaneous pain is a major complaint of patients suffering from many forms of acute and chronic pain. Ongoing activity in nociceptors is a major driver of ongoing pain in humans, and similar OA is observed in isolated nociceptors in rodent pain models. Three neurophysiological alterations contribute to OA in the absence of sensory generator potentials – depolarization of RMP, hyperpolarization of AP

threshold, and enhancement of depolarizing spontaneous fluctuations of the membrane potential (DSFs). OA can be enhanced acutely in nociceptors by inflammatory mediators released by immune and glial cells, such as serotonin, which will be discussed in detail in later chapters. Cyclic AMP is a major mediator of intracellular signal transduction in response to a number of hormones, neurotransmitters, and inflammatory mediators, and cAMP and its effectors PKA and EPAC are known contributors to persistent OA after spinal cord injury (Bavencoffe et al., 2016; Berkey et al., 2020; Garza Carbajal et al., 2020). However, neuromodulators and cellular signaling pathways that increase DSF amplitude and thereby promote OA acutely in nociceptors are not known.

Chapter 2

Materials and Methods

Note: Text is partially reprinted from E. R. Lopez, A. Garza Carbajal, J. B. Tian, A. Bavencoffe, M. X. Zhu, C. W. Dessauer, and E. T. Walters. 2021. Serotonin enhances depolarizing spontaneous fluctuations, excitability, and ongoing activity in isolated rat DRG neurons via 5-HT_4 receptors and cAMP-dependent mechanisms. *Neuropharmacology* 184: 108408. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Elsevier Ltd.

2.1. Animals

All procedures were in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200-300 g, 2 per cage) were maintained in the McGovern Medical School animal research facility under a 12:12 h reversed light/dark cycle, and experiments were performed during the dark phase. Rats had access to food and water ad libitum.

2.2. Dissociation and culture of DRG neurons

Rats were euthanized by intraperitoneal injection of pentobarbital/phenytoin (0.9 mL; Euthasol, Virbac AH, Inc) followed by transcardial perfusion of ice-cold phosphate-buffered saline (PBS, Sigma-Aldrich). DRG from spinal levels T8 to L6 (for pharmacological experiments) or L4 to L6 (for plantar incision experiments, keeping ipsilateral and contralateral DRG separate) were excised and digested with trypsin (0.3 mg/mL, Worthington) and collagenase D (1.4 mg/mL, Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) for 40 min at 34°C. Digested DRG were washed twice with warm (37°C) DMEM followed by mechanical trituration in DMEM with fire-polished Pasteur pipettes. Dissociated cells were seeded on glass coverslips (for electrophysiological recordings or Ca^{2+} imaging) or a 96-well cell culture microplate (for high content microscopy) coated with poly-L-ornithine solution (0.01%). Cell cultures were incubated overnight (5% CO_2, 95% humidity, 37°C) in DMEM without serum, growth factors, or other supplements, and experiments were performed 18-28 h post-dissociation.

2.3. Whole-cell recordings from dissociated DRG neurons

Small- to medium-sized DRG neurons (soma diameter ≤30 μm and input capacitance ≤45 pF, to exclude most LTMRs and proprioceptors and patch predominantly nociceptors) that were not in visible contact with other neurons or debris were recorded in whole-cell configuration at room temperature using either a Zeiss Axiovert 200M with 40X magnification and a HEKA EPC10 amplifier or an Olympus IX-71 with 40X magnification and a MultiClamp 700B amplifier (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries with a 1.5 mm outer diameter and 0.86 mm inner diameter (Sutter Instrument Co) using a Sutter P-97 Flaming/Brown Micropipette Puller followed by polishing. Patch pipettes were filled with an intracellular-like solution (ICS; in mM: 134 KCl, 1.6 $MgCl_2$, 13.2 NaCl, 3 EGTA, 9 HEPES, 4 Mg-ATP, and 0.3 Na-GTP, adjusted to pH 7.2 with KOH and osmolarity 300 with sucrose) and had an electrode resistance of 2-10 MΩ. The extracellular solution (ECS) contained (in mM): 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose and was adjusted to pH 7.4 with NaOH and osmolarity 320 with sucrose. After forming a tight seal, the membrane was ruptured to establish whole-cell configuration under voltage clamp, and membrane resistance and input capacitance were measured. The liquid junction potential (~4.3 mV) was not corrected.

To measure OA at rest, neurons were recorded in current clamp with no current injection for a minimum of 1 min. Next, current was injected to maintain the membrane potential at approximately -60 mV while a series of 2-ms depolarizing current injections (200 ms sweep interval, +10 pA or +20 pA increments) were used to measure AP voltage threshold. This was followed by a series of 2-s depolarizing current injections

(4-s sweep intervals, increments of +5 pA, +10 pA, or +20 pA) to measure rheobase and to provide an additional measure of AP voltage threshold. Each of these procedures yields consistent estimates of AP voltage threshold (Odem et al., 2018); my primary measure was the AP threshold determined with 2-s pulses. To measure OA at -45 mV, current was injected to maintain a membrane potential of approximately -45 mV for at least 30 s. DSFs were quantified using our algorithm SFA.py coded in Python v3.5.2 (Python Software Foundation), as described previously (Odem et al., 2018), from the 30-s traces recorded at -45 mV. In addition to subthreshold DSFs identified by the program, suprathreshold DSFs were included, for which the amplitude was calculated as the difference between the start membrane potential of the event and the AP threshold. DSFs were not quantified from cells exhibiting firing frequencies above ~1.5 Hz.

For neurons treated with serotonin (5-HT, serotonin hydrochloride, Abcam and Sigma-Aldrich) or prucalopride (Sigma-Aldrich), recordings were made from cells that had been pre-exposed to the drug (at least 5 min), and the drug was continuously present during the recordings (up to 60 min). There was no apparent change in the effects on the properties measured over this period. To test the effects of 5-HT receptor antagonists, PKA inhibitors, EPAC inhibitors, or a hyperpolarization-activated cyclic-nucleotide-gated channel (HCN) inhibitor, coverslips were incubated at 37°C in ECS containing GR113808, ketanserin-tartrate salt, myristoylated PKI 14-22 amide (all from Sigma-Aldrich), RS127445, granisetron, SB269970, CE3F4, H-89, ZD7288 (all from Cayman Chemical), or ESI-05 (synthesized as described by Chen et al., 2013) for a minimum of 5 min before being transferred to the recording chamber containing the inhibitor plus 5-HT in ECS. With the exception of 8-Br-cAMP, all drugs were added to

the ECS. The chambers were not continuously perfused. 8-Br-cAMP (Sigma-Aldrich) was diluted in ICS for intracellular delivery via the patch pipette (resistance of 2-6 MΩ). For intracellular dialysis of 8-Br-cAMP, after obtaining whole-cell configuration, the cell was maintained at -60 mV under voltage clamp for at least 3 min before switching to current clamp and proceeding with data collection.

All data shown are from non-accommodating (NA) type neurons, excluding the rapidly accommodating (RA) type neurons (Odem et al., 2018). Thus, all electrophysiological data analyzed includes probable nociceptors (based on soma size) that are capable of repetitive firing. Additionally, our previous study using the same culture conditions and size selection criterion indicated 70% of NA neurons patched were sensitive to capsaicin and thus presumed $TRPV1^+$ (Odem et al., 2018).

2.4. High content microscopy and subpopulation analysis

The following materials were used. Primary antibodies: chicken anti-PGP9.5 (1:4000, Novus Biologicals, # NB110-58872), rabbit monoclonal anti-phospho RII (S99) (1:1000, clone 151, Abcam, # ab32390), and mouse anti-CGRP (1:1000, Santacruz #SC-57053). Secondary antibodies (all 1:1000): goat anti-chicken-DyLight 755, goat anti-rabbit 568, donkey anti-mouse AF 647. Isolectin B4-FITC (1:1500, MilliporeSigma, #L2895) and DAPI.

Following pharmacological treatments at 37°C, cells were fixed with 4% paraformaldehyde for 10 min then washed with PBS. Cells were then blocked for one hour at room temperature with blocking solution containing 1% bovine serum albumin and 0.075% Triton X-100 in PBS followed by incubation with primary antibodies in

blocking solution at 4°C overnight. After washing, cells were incubated with secondary antibodies and DAPI in blocking solution or with isolectin B4 (IB4) in IB4 buffer (100 µM each $MgCl_2$, $CaCl_2$, and $MnCl_2$ in PBS) at room temperature in the dark for one hour followed by three final PBS washes.

Plates were imaged using a Cellomics CX5 microscope (Thermo Scientific) with a 10x objective following modified protocols described by Isensee et al. (2018, 2017, 2014). Cellomics software package (Thermo Scientific) was used to analyze 1104x1104 pixel images. Neurons were identified by PGP 9.5 staining intensity. When appropriate, bleed through between channels was compensated with raw fluorescence data from fluorescence controls using the slope determined by linear regression (Prism, GraphPad) as described by Roederer, 2002.

One- and two-dimensional density plots were generated using FlowJo (Becton Dickinson). Gating of subpopulations, based on neuronal soma area, CGRP expression, and IB4 binding as established previously by Garza Carbajal et al. (2020), was performed by setting thresholds at local minima of probability in 2D plots and corroborated by 3D cluster analysis. Individual cells used to perform the cluster analysis were normalized between the minimal (0.001%) and maximal (0.999%) fluorescence levels per channel. Cluster analysis (k-medians) was performed using Cluster 3.0 software (de Hoon et al., 2004). Three-dimensional (3D) plots were constructed using Plotly (Chart Studio). Data analysis and graph plots were performed using Prism.

2.5. Ca^{2+} imaging

Overnight cultured DRG neurons were loaded with 2 µM Fura-2AM (TEFLabs.com) in DMEM in the cell culture incubator for 1 hour. Neurons on coverslips

were placed in a diamond-shaped RC-40 low profile bath chamber on a QE-1 quick exchange platform (both from Warner Instruments) and were constantly perfused with the same ECS used for electrophysiological recording. Images were acquired on a live cell fluorescence system, including Nikon Eclipse TE2000-U microscope with 40X oil objective lens and a Sutter Instrument Lambda DG-4 ultra-high-speed wavelength switching system controlled by SlideBook software v5.0. Fura-2 fluorescence signals were excited alternately at 340 nm and 380 nm every 3 seconds and detected at 510 nm emission. Serotonin (300 nM or 10 µM) or capsaicin (500 nM, Sigma-Aldrich) diluted in ECS or KCl (60 mM, made by substituting 57 mM NaCl from ECS with equimolar KCl) was applied to neurons through whole chamber perfusion by a peristaltic pump at a flow rate of approximately 2 ml/min. Serotonin was applied 90-120 secs following 2 min baseline recording. Capsaicin was applied following 5-HT stimulation to identify capsaicin-sensitive nociceptors. KCl was applied at the end of each recording to confirm responses from live neurons. Fura-2 fluorescence ratios (F340/F380) were analyzed using SlideBook to estimate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes.

2.6. Surgical procedures

Rats were anesthetized with isoflurane for plantar incision as described previously by Brennan, et al. (Brennan et al., 1995). Anesthesia was induced by placing the rat in a chamber filled with 4% isoflurane in air followed by 2% isoflurane via a nose cone maintained during surgery. The left hind paw was sterilized with alternating iodine swabs and alcohol pads. Beginning 0.5-1 cm from the proximal edge of the heel, a ~2 cm longitudinal incision was made through the skin and fascia. The flexor digitorum

brevis muscle was then elevated and incised longitudinally, leaving intact the muscle origin and insertion. The skin was closed with two absorbable sutures.

2.7. Measurement of guarding behavior

Rats were placed individually on an elevated metal mesh (grid 6 x 6 mm) and enclosed by a clear plastic cage top (20.5 x 20.5 x 13.5 cm). Both hind paws were closely observed for a 1-min period every 4 min for 48 min or every 3 min for 36 min, depending on the number of rats in each group. For each 1-min observation period a score of 0, 1, or 2 was given for each paw according to its position during the majority of the period. As described previously (Xu and Brennan, 2010), a score of 0 was given if the incised area was touching and being blanched or distorted by the mesh. A score of 1 was given if the incised area was touching the mesh without being blanched or distorted. A score of 2 was given if the incised area was completely off of the mesh. A sum score was obtained for each hind paw by adding the 12 scores observed during the 48-min or 36-min testing period. The guarding score was obtained by subtracting the score of the uninjured paw from that of the injured paw.

2.8. Statistical analysis

All data sets were tested for normality with the Shapiro-Wilk and D'Agostino and Pearson tests. Normally distributed data were analyzed by parametric tests: t-test or 1- or 2-way ANOVA followed by Dunnett's or Tukey's multiple comparisons tests using Prism v8 (GraphPad Software, Inc). Non-normally distributed data were analyzed by non-parametric tests: Mann-Whitney or Kruskal-Wallis (GraphPad Software, Inc). If any data set within an analysis failed to pass either normality test, non-parametric statistics

were performed. Comparisons of incidences were made with Fisher's exact test. All tests were two-tailed, with one exception. The dose-response relationship for 5-HT and OA was tested with a one-tailed Fisher's exact test on the basis of prior observations indicating that the incidence of OA, which is low under our control conditions, would be increased but not decreased by 5-HT treatment. All t-tests were unpaired unless otherwise stated. Dose-response curves were fitted using Prism v8.

2.9. RNA sequencing

Seven days after deep tissue plantar incision surgery, the L4-6 DRG were collected from naïve and incised rats, keeping the left (ipsilateral) and right (contralateral) sides separate. All surgeries took place in the morning, and tissue collection was carried out in the afternoon on postoperative day 7. To ensure an adequate amount of tissue for RNA extraction, DRG were pooled from 2 rats for each sample. Thus, each sample contained 6 DRG – unilateral L4-6 from 2 rats. Each condition had 4 replicates, so each condition was represented by 8 rats. Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen).

RNA quantification and qualification, library preparation, sequencing, and bioinformatics analysis were performed by Novogene Co., Ltd in Sacramento, CA. Briefly, RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN), and integrity and quantification were assessed using the RNA Nano 600 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). A total of 1 µg RNA per sample was used as input materials for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). Library quality

was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina), then library preparations were sequenced on an Illumina platform and paired-end reads were generated. Raw reads (FASTQ format) were processed through fastp to obtain clean reads by removing reads containing adapter and poly-N sequences and reads with low quality. Downstream analyses were based on the clean data with high quality. Paired-end clean reads were mapped to the reference genome (*Rattus norvegicus*) using HISAT2 software. Featurecounts was used to count the read numbers mapped of each gene, including known and novel genes, and FPKM of each gene was calculated based on the length of the gene and reads count mapped to the gene. Differential expression analysis between two groups was performed using DESeq2 R package. The resulting p values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate.

Chapter 3

5-HT enhances DSFs, excitability, and OA in isolated rat DRG neurons

Note: Figures and text are partially reprinted from E. R. Lopez, A. Garza Carbajal, J. B. Tian, A. Bavencoffe, M. X. Zhu, C. W. Dessauer, and E. T. Walters. 2021. Serotonin enhances depolarizing spontaneous fluctuations, excitability, and ongoing activity in isolated rat DRG neurons via 5-HT_4 receptors and cAMP-dependent mechanisms. *Neuropharmacology* 184: 108408 and M. A. Odem, A. G. Bavencoffe, R. M. Cassidy, E. R. Lopez, J. Tian, C. W. Dessauer, and E. T. Walters. 2018. Isolated nociceptors reveal multiple specializations for generating irregular ongoing activity associated with ongoing pain. *Pain* 159 (11): 2347. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Wolters Kluwer Health, Inc. and Elsevier Ltd.

3.1. Introduction

As described in Chapter 1, pro-inflammatory mediators contribute to pain sensitization during injury and inflammation. One such mediator of interest is serotonin (5-hydroxytryptamine, 5-HT). Serotonin is well known for its important role as a neurotransmitter in the central nervous system in regulating mood and behavior. However, 5-HT mediates a wide variety of physiological functions both in the central nervous system and in the periphery, including gut motility, pain and inflammation, and other processes (Berger et al., 2009; Feldman, 2004; Sommer, 2004; Yadav, 2013). These two pools of 5-HT, one in the blood and periphery and one in the brain, function like two distinct molecules (Mann et al., 1992). Most of the 5-HT present in the periphery is synthesized in enterochromaffin cells of the gut (Gershon and Tack, 2007), where the rate-limiting step is the conversion of L-tryptophan to L-5OH-tryptophan by tryptophan hydroxylase (TPH). L-5OH-tryptophan is then converted to 5-HT by an aromatic L-amino acid decarboxylase (Mann et al., 1992; Walther et al., 2003). TPH1 carries out serotonin synthesis in the periphery, whereas TPH2 is responsible for serotonin synthesis in serotonergic neurons in the brain (Walther et al., 2003).

Serotonin was linked to pain and nociceptor hyperexcitability in a number of previous studies. Intradermal application of 5-HT produces hyperalgesia in mice and rats (Lin et al., 2011; Taiwo and Levine, 1992) and pain in humans (Schmelz et al., 2003). Serotonin application to dissociated DRG neurons can decrease the current needed to elicit an AP, increase the AP amplitude (Cardenas et al., 2001) and increase AP firing frequency during depolarizing current steps (Salzer et al., 2016). Several studies of 5-HT effects on nociceptor excitability have examined the effects of micromolar concentrations, often using relatively fast perfusion. Because exposure of

nociceptor somata to inflammatory mediators like serotonin may occur through prolonged exposure to low concentrations in the blood and/or cerebrospinal fluid (CSF), it is important to also examine the effects of relatively low concentrations over longer time periods.

3.2. Results

3.2.1. Dose-dependent relationship of the effects of 5-HT on hyperexcitability

To examine the dose-response relationship of 5-HT effects on DRG neurons under conditions of relatively prolonged exposure, I examined the effects of different concentrations (from 0.1 nM to 10 µM) of 5-HT on functionally important excitability properties in probable nociceptors exposed to 5-HT for ~5-60 min. Consistent with earlier observations that low and high doses of 5-HT enhance a voltage-gated Na^+ channel conductance (Cardenas et al., 1997; Gold et al., 1996), prolonged 5-HT treatment hyperpolarized the AP voltage threshold (Figure 3A; 1-way ANOVA, $F(6,76) = 2.28$, $p = 0.045$) at 10 nM and 100 nM compared to the vehicle control. Serotonin increased the incidence of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs recorded while the membrane potential was held at approximately -45 mV under current clamp (Figure 3B; Kruskal-Wallis, $p = 0.0024$ and $p = 0.018$, respectively) at concentrations of 10 nM and 100 nM. The greatest increase in the incidence of OA at -45 mV occurred at 100 nM 5-HT (Figure 3C; 1-sided Fisher's exact test, $p = 0.005$). There were trends toward increased OA incidence with concentrations as low as 10 nM ($p = 0.065$) and as high as 10 µM 5-HT ($p = 0.11$). Furthermore, there was an overall effect of 5-HT across all doses in reducing the rheobase during 2-s depolarizing test pulses, although individual doses showed no significant effects (Figure 3D; Kruskal-

Wallis, p = 0.048). None of the tested 5-HT concentrations produced significant effects on either RMP (Figure 3A; 1-way ANOVA, $F(6,76) = 1.61$, $p = 0.16$) or OA recorded at RMP (Figure 3C).

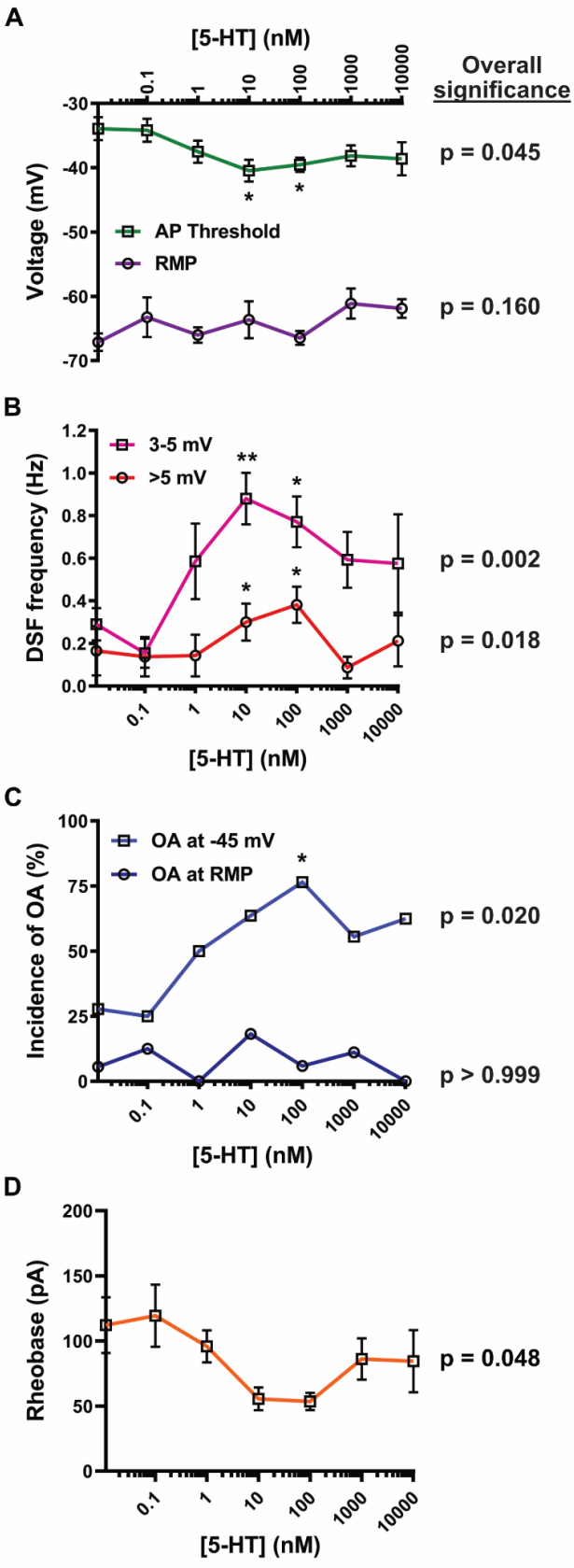


Figure 3. Dose-response effects of 5-HT on RMP, AP threshold, DSFs, and OA. DRG neurons were treated with 5-HT or vehicle before and during recording. Each concentration is represented by a minimum of 8 cells from at least 2 rats. P values listed under overall significance indicate the result of ANOVA, Kruskal-Wallis, or Fisher's exact tests assessing the presence of an overall effect across all doses. (**A**) 5-HT effects on RMP and AP threshold. Mean ± SEM. * $p < 0.05$. (**B**) 5-HT effects on the frequency of medium-amplitude (3-5 mV) and large-amplitude (>5 mV) DSFs during 30-s depolarization to -45 mV. Mean ± SEM. * $p < 0.05$, ** $p < 0.01$. (**C**) 5-HT effects on the incidence of OA measured during a ≥ 60-s recording at RMP and then during a 30-s depolarization to -45 mV. P values indicate the result of Fisher's exact tests comparing no 5-HT to all 5-HT concentrations combined. * $p < 0.0083$ (after Bonferroni correction for 6 comparisons). (**D**) 5-HT effects on rheobase. Mean ± SEM. Kruskal-Wallis test revealed a significant overall difference, but Dunn's test did not reveal differences at individual doses.

3.2.2. Exposure to 100 nM 5-HT potentiates OA in probable nociceptors, in part by enhancing DSFs

The effect of 100 nM 5-HT, the dose that yielded the strongest potentiation of OA (Figures 3C, 4A), on DSFs was examined in detail. During 30-s depolarization to -45 mV both the frequency of large (>5 mV amplitude) DSFs and frequency of APs were strongly increased by 5-HT (Figure 4B; Mann-Whitney tests, $p < 0.0001$). The mean amplitude of all DSFs (at least 1.5 mV amplitude) was increased in neurons treated with 5-HT (Mann-Whitney, $p < 0.0001$), and DSFs were largest in cells that had OA at -45 mV (Figure 4C; Mann-Whitney, $p < 0.0001$). Representative traces illustrating DSFs and OA in probable nociceptors held at -45 mV are shown in Figure 4D. As illustrated by raster plots (Figure 5A1,B1), DSFs occurred randomly in the control and 5-HT-treated conditions, and 5-HT increased the frequency of medium-amplitude DSFs (Mann-Whitney, $p < 0.001$), which rarely triggered APs (Figure 5A2), as well as the frequency of large-amplitude DSFs (Mann-Whitney, $p < 0.0001$), which frequently reach AP threshold (Figure 5B2; Fisher's exact test, $p < 0.05$).

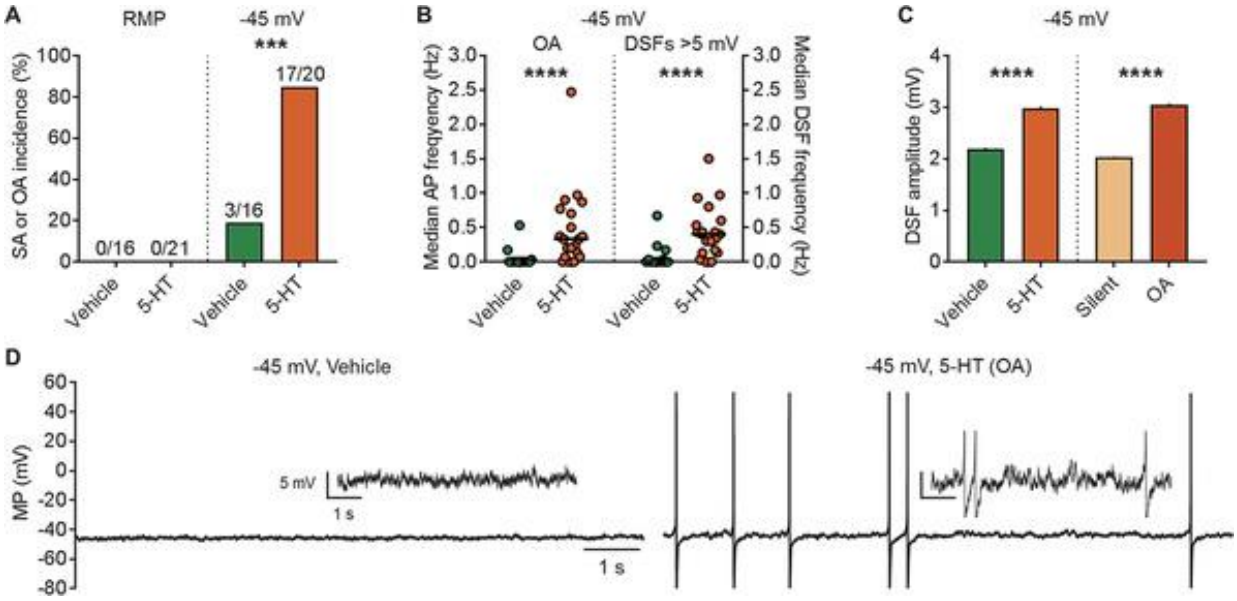


Figure 4. Potentiation by 5-HT of OA in probable nociceptors. Dorsal root ganglion neurons from naive rats (n = 4) were treated with vehicle or 100-nM 5-HT for 10 to 30 minutes before and during each recording. After measurement of any SA, extrinsically driven OA was modeled by depolarization to -45 mV under current clamp for 30 to 60 seconds. (A) Pretreatment with 5-HT did not induce OA at RMP but significantly increased OA at -45 mV (the Fisher exact test). (B) In neurons tested at -45 mV, 5-HT significantly increased AP frequency during OA and large DSF frequency. Black lines-medians. (C) 5-HT increased the amplitude of DSFs measured at -45 mV, and the neurons with OA showed larger DSFs than silent neurons. Depolarizing spontaneous fluctuation sample sizes left to right: 1360, 2113, 1256, and 2217. Data are shown as mean +/- SEM. *** $p < 0.001$ **** $p < 0.0001$. (D) Representative recordings SFs and OA at -45 mV after treatment with vehicle or 5-HT. Insets: enlarged sections from each trace. AP, action potential; DSF, depolarizing spontaneous fluctuation; MP, membrane potential; OA, ongoing activity; RMP, resting membrane potential; SA, spontaneous activity. Data collected by Elia R. Lopez, DSF analysis and figure by Max A. Odem.

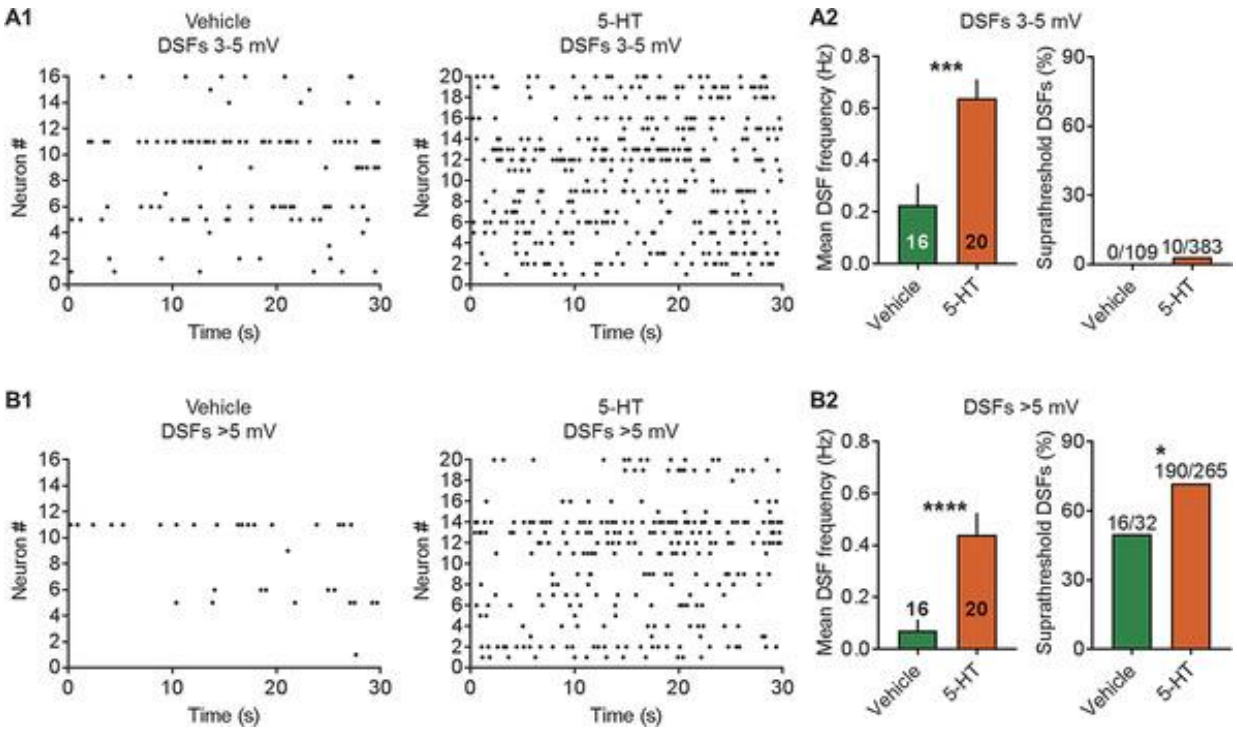


Figure 5. Treatment with 5-HT before and during recording at -45 mV increases the number of medium amplitude and large DSFs in probable nociceptors from naive rats. (A1, B1) Raster plots of medium amplitude and large DSFs during depolarization to -45 mV. Each row represents one neuron and each point a single DSF. (A2, B2) At -45 mV, 5-HT increased the number of medium amplitude and large DSFs, and the percentage of DSFs evoking APs. Bars represent the mean +/- SEM or fraction in total sample, and significance was tested using Mann-Whitney U or Fisher exact tests. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Neurons are from the naive rats (n = 4) used in Figure 6. AP, action potential; DSF, depolarizing spontaneous fluctuation. Data collected by Elia R. Lopez, DSF analysis and figure by Max A. Odem.

3.3. Conclusions

These results show that OA and two of its major electrophysiological mechanisms (increased DSF amplitude and decreased AP voltage threshold) in probable nociceptors are sensitive to relatively low concentrations of 5-HT. In addition, the results confirm

that, while these 5-HT concentrations enhance AP generation by at least two general electrophysiological alterations, this enhancement is only expressed when the membrane potential is maintained at a relatively depolarized level within the physiological range. Thus, prolonged (5-60 min) application of 100 nM 5-HT combined with an extrinsic source of depolarization (to -45 mV) for 30-60 sec provides a useful model for defining cell signaling mechanisms by which OA in nociceptors is enhanced acutely.

Chapter 4

5-HT potentiates OA in DRG neurons via 5-HT_4 receptors

Note: Figures and text are partially reprinted from E. R. Lopez, A. Garza Carbajal, J. B. Tian, A. Bavencoffe, M. X. Zhu, C. W. Dessauer, and E. T. Walters. 2021. Serotonin enhances depolarizing spontaneous fluctuations, excitability, and ongoing activity in isolated rat DRG neurons via 5-HT_4 receptors and cAMP-dependent mechanisms. *Neuropharmacology* 184: 108408. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Elsevier Ltd.

4.1. Introduction

While 5-HT-stimulated cell signaling that may lower AP threshold in nociceptors has been explored (Cardenas et al., 2001; Gold et al., 1996), cell signaling pathways that enhance DSFs and OA acutely have not been described. Multiple signaling pathways have been implicated in 5-HT effects on nociceptor excitability and pain, potentially linked to numerous 5-HT receptors expressed in the DRG, including those coupled to G_s, G_i, and G_q, as well as 5-HT-gated cation channels (Chen et al., 1998; Lin et al., 2011; Nicholson et al., 2003; Ohta et al., 2006). My objective was to define the predominant cell signaling mechanism by which 5-HT enhances DSFs and associated OA. Because 5-HT is known to strongly activate PKA in nociceptors (Isensee et al., 2017) and our previous studies linked PKA and EPAC activity to chronic pain-related OA (Bavencoffe et al., 2016; Berkey et al., 2020), I hypothesized that G_s-coupled 5-HT receptors (5-HTR) mediate the enhancement of DSFs, reduction of AP threshold, and consequent potentiation of OA by 5-HT.

4.2. Results

4.2.1. 5-HT_4 receptors are required for potentiation of DSFs and OA induced by 5-HT

To begin to elucidate the cell signaling pathways by which 5-HT modulates DSFs and AP threshold, and their effects on OA, I asked which 5-HT receptors are required. Selective antagonists of 5-HT receptors known to be expressed in the DRG were added to the 5-HT solution. The incidence of OA recorded at -45 mV in the presence of 100-300 nM 5-HT was blocked by the selective 5-HT_4 receptor antagonist GR113808 (Figure 6A, B; Fisher's exact test, $p = 0.0002$) but not by the other 5-HT receptor antagonists

tested. GR113808 reduced the frequencies of medium-amplitude (3-5 mV; Kruskal-Wallis, $p = 0.01$) and large-amplitude (>5 mV; Kruskal-Wallis, $p = 0.0007$) DSFs at -45 mV during exposure to 5-HT (Figure 6C). Additionally, treatment with GR113808 resulted in a more depolarized AP threshold in the presence of 5-HT compared to vehicle/5-HT (Figure 6D; Kruskal-Wallis, $p = 0.024$). Moreover, GR113808 prevented reduction of the rheobase by 5-HT (Figure 6E; Kruskal-Wallis, $p = 0.0099$). Selective antagonists of $5\text{-HT}_{2A/2C}$ (ketanserin), 5-HT_{2B} (RS127445) or 5-HT_{3} (granisetron) had no effect on any of these measures (Figure 6A, C, D), and there was no significant effect on RMP by any of the agents tested (Figure 6F; Kruskal-Wallis, $p = 0.1963$).

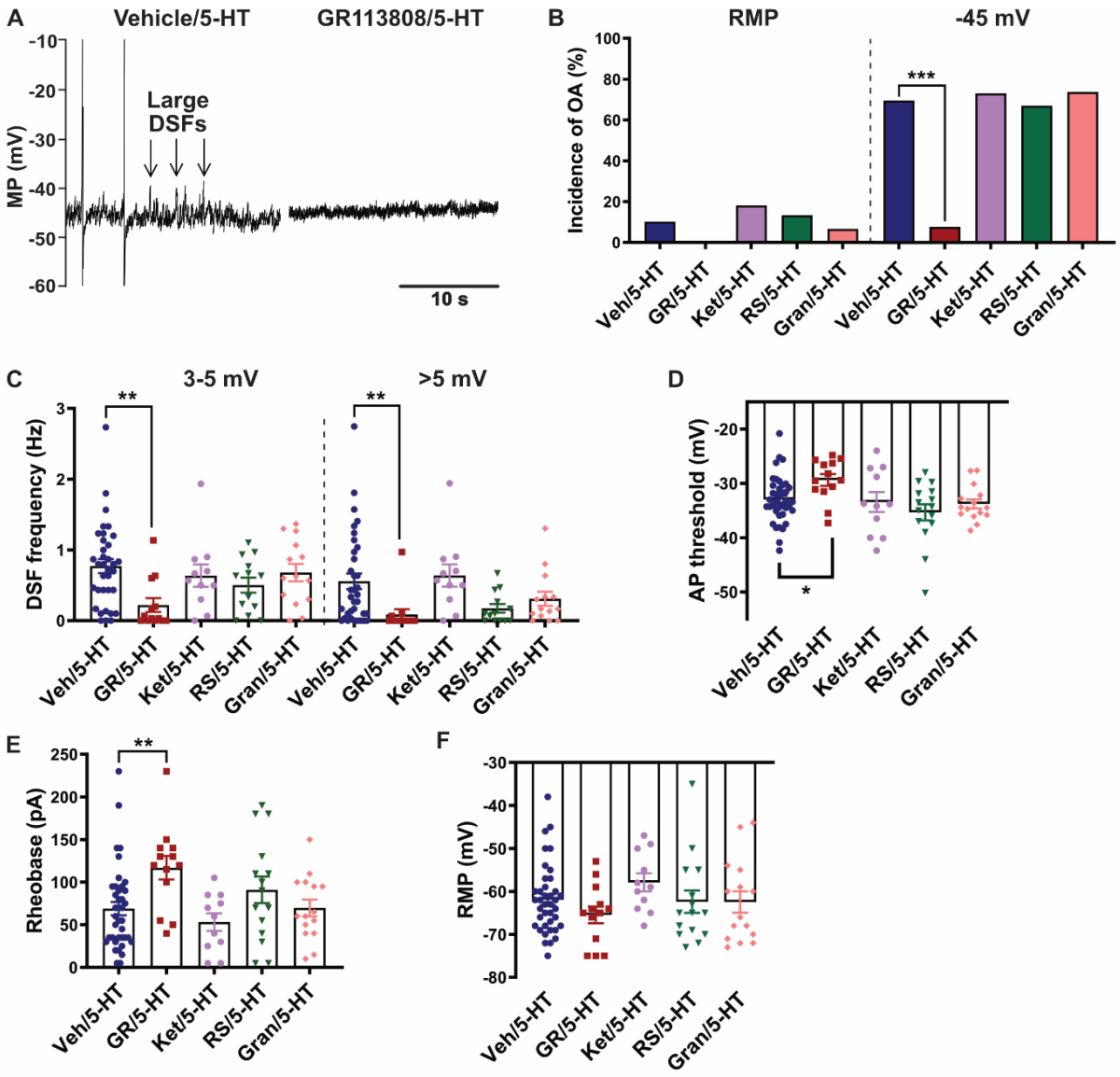


Figure 6. 5-HT$_4$ receptor is required for potentiation of DSFs and OA by 5-HT. Cells were pre-treated for 5-15 min with 1 μM of the 5-HTR antagonist or vehicle control before cells were patched in the presence of the antagonist plus 100-300 nM 5-HT. (**A**) Representative recordings at -45 mV in different neurons exposed to vehicle/5-HT or GR113808/5-HT. Note the two APs (OA) and large DSFs in the left panel. MP = membrane potential. (**B**) 5-HTR antagonist effects on the incidence of OA during exposure to 5-HT. OA was measured at RMP for ≥60 s then during depolarization to -45 mV for 30 s. *** $p < 0.00025$ (after Bonferroni correction for 4 comparisons). (**C-E**) 5-HTR antagonist effects on the frequency of medium- (3-5 mV) and large-amplitude (> 5 mV) DSFs during 30-s depolarization to -45 mV (**C**), AP threshold (**D**), rheobase (**E**), and RMP (**F**) in the presence of 5-HT. Mean ± SEM. * $p < 0.05$. ** $p < 0.01$.

In high-content imaging studies using PKA-pRII as a surrogate readout of PKA activation (Isensee et al., 2014), the selective 5-HT_4 antagonist GR113808 blocked PKA activation in response to 100 nM 5-HT in a dose-dependent manner (Figure 7; IC50 = 5.55 nM; 2-way ANOVA, $F(16,63) = 3.18$, $p = 0.0005$), confirming a previous report (Isensee et al., 2017). Surprisingly, although there is evidence for expression of the G_s-coupled 5-HT_7 receptor in rodent and human DRG neurons (Chen et al., 1998; Lin et al., 2011; Morita et al., 2015; Ohta et al., 2006), and the selective 5-HT_7 agonist LP44 can evoke AP firing, albeit at a high concentration (Morita et al., 2015), I found that a selective antagonist of 5-HT_7 (SB269970) had no appreciable effect on PKA activation by 5-HT (Figure 7). As expected, 5-$HT_{2A/2C}$ (ketanserin), 5-HT_{2B} (RS127445) and 5-HT_3 (granisetron) antagonists had no significant effect on PKA activation (Figure 7). These results suggest that, at these 5-HT concentrations, the G_s-coupled 5-HT_7 receptor does not play a role in PKA activation in primary DRG neurons, and the G_q-coupled receptors (5-HT_2) and 5-HT-gated cation channels (5-HT_3) do not alter PKA activation produced by activation of the 5-HT_4 receptor.

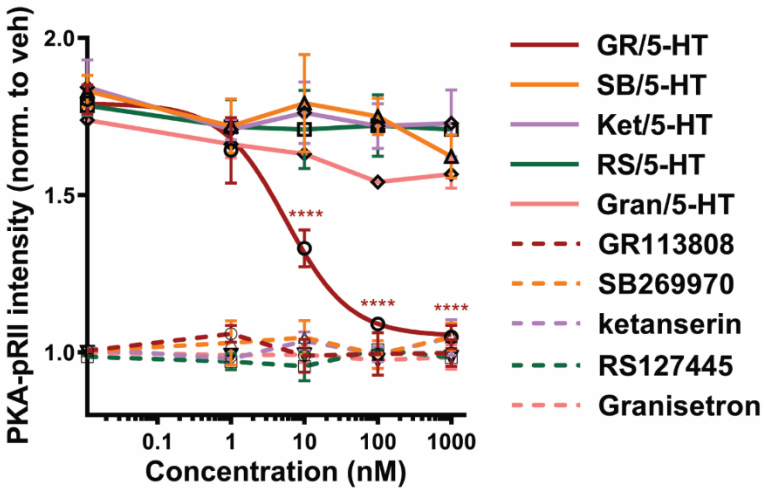


Figure 7. PKA activation measured by PKA-pRII staining in rat DRG neurons. Different concentrations of antagonists were applied 30 min prior to 5-min co-application of the antagonist and 100 nM 5-HT. Each data point represents the mean ± SEM of 3-5 separate experiments. **** $p < 0.0001$.

To further address whether G_q-coupled 5-HT_2 receptors might contribute to nociceptor responses to 5-HT under these potentially desensitizing electrophysiological testing conditions (relatively slow delivery and continuing exposure to 5-HT for at least several minutes), we measured Ca^{2+} responses to 5-HT in probable nociceptors using Fura-2. Example recordings from a neuron that failed to respond to 90-120 s superfusion with either 300 nM or 10 µM 5-HT and another neuron that showed a weak response to the higher concentration are shown in Figure 8A. Only 5.6% (2/36) and 9.7% (3/31) of neurons responsive to capsaicin (i.e. probable nociceptors) showed detectable increases in $[Ca^{2+}]_i$ during superfusion with 300 nM or 10 µM 5-HT, respectively (Figure 8B). Among the capsaicin-insensitive neurons, 0% (0/11) and 33.3% (2/6) showed an increase in $[Ca^{2+}]_i$ in response to 300 nM or 10 µM 5-HT, respectively. These observations indicate that increasing 5-HT concentration relatively slowly (over tens of

seconds under our superfusion conditions) elicits Ca^{2+} responses in only a small subset of DRG neurons (4.3 and 13% of all neurons tested in 0.3 and 10 µM 5-HT, respectively). Taken together, these data indicate that DSFs and associated OA are enhanced in a sustained manner by relatively low concentrations of 5-HT that fail to evoke Ca^{2+} responses in most of the neurons sampled, and these effects are mediated primarily by the G_s-coupled 5-HT_4 receptor.

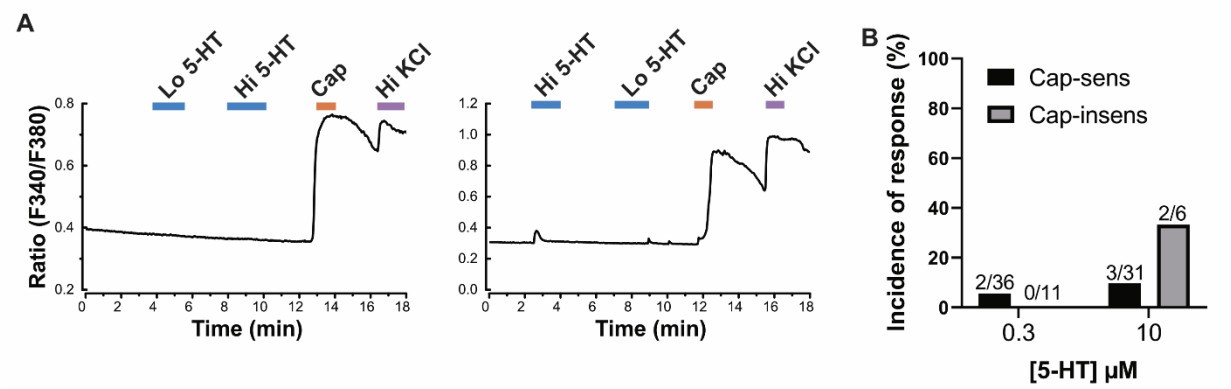


Figure 8. Ca^{2+} responses to 5-HT measured by Fura-2. (**A**) Representative traces of Fura-2 ratio changes used to indicate increases in $[Ca^{2+}]_i$ during superfusion of 300 nM or 10 µM 5-HT in single capsaicin-sensitive neurons. (**B**) Incidence of 5-HT-associated increases in $[Ca^{2+}]_i$ as measured with Fura-2. Cap-sens = capsaicin sensitive. Cap-insens = capsaicin-insensitive. Lo 5-HT = 300 nM 5-HT. Hi 5-HT = 10 µM 5-HT. Cap = 500 nM capsaicin. Hi KCl = 60 mM KCl. Data collected by Jin Bin Tian.

4.2.2. Selective 5-HT_4 activation can induce hyperexcitability in DRG neurons

Is selective activation of the 5-HT_4 receptor sufficient to enhance DSFs and promote OA in small DRG neurons? To answer this question, I used a selective 5-HT_4 agonist, prucalopride, and compared the dose dependence of its activation of PKA to the activation of PKA by 5-HT, along with the inhibition of these effects by an effective

dose of the 5-HT_4 receptor antagonist GR113808 (Figure 9). Activation of PKA by prucalopride and 5-HT exhibited a similar dose dependence, with an EC_{50} of 4.53 (95% confidence interval (CI) = 1.78 – 11.45, R2 = 0.89) for 5-HT and 10.23 nM (95% CI = 4.06 – 25.61, R2 = 0.89) for prucalopride. A 2-way ANOVA revealed significant overall effects of dose ($F(5,48) = 38.83$, $p < 0.0001$) and drug ($F(3,48) = 56.32$, $p < 0.0001$). Importantly, GR113808 (1 µM) significantly reduced PKA activation by both 5-HT and prucalopride at all doses ≥ 10 nM and ≥ 100 nM, respectively (Figure 9).

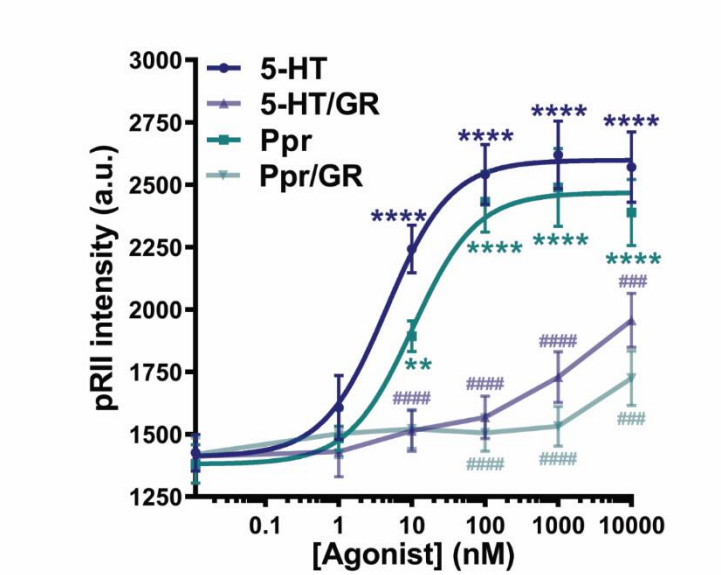


Figure 9. Dose-dependent PKA activation by 5-HT and the 5-HT_4 agonist prucalopride. Vehicle (DMSO) or 1 µM GR113808 was co-applied with 5-HT or prucalopride for 5 min. Each data point represents the mean ± SEM of 3 separate experiments. Asterisks indicate significant difference of the agonist dose compared to no agonist. Hash marks indicate significant difference in the presence of antagonist compared to no antagonist. ** $p < 0.01$, ### $p < 0.001$, ****/#### $p < 0.0001$. Ppr, prucalopride; GR, GR113808.

Exposure to prucalopride (300 nM, ~5-60 minutes), like 5-HT, had no effect on RMP (Figure 10A; Mann-Whitney, $p = 0.79$) and did not induce OA at rest (Figure 10B). Prucalopride strongly increased the incidence of OA upon depolarization to -45 mV (Figure 10B, C; Fisher's exact test, $p = 0.0002$). The modest experimental depolarization also revealed increases in mean DSF amplitude (Figure 10C, D; Mann-Whitney, $p = 0.0006$) and increased frequencies of medium- and large-amplitude DSFs (Figure 10E; Mann-Whitney, $p = 0.0022$ and $p = 0.0014$, respectively), similar to that observed with comparable concentrations of 5-HT (Figure 3, Figure 5). Prucalopride significantly hyperpolarized AP voltage threshold (Figure 10F; Mann-Whitney, $p = 0.0036$) and markedly reduced rheobase (Figure 10G; Mann-Whitney, $p = 0.0024$).

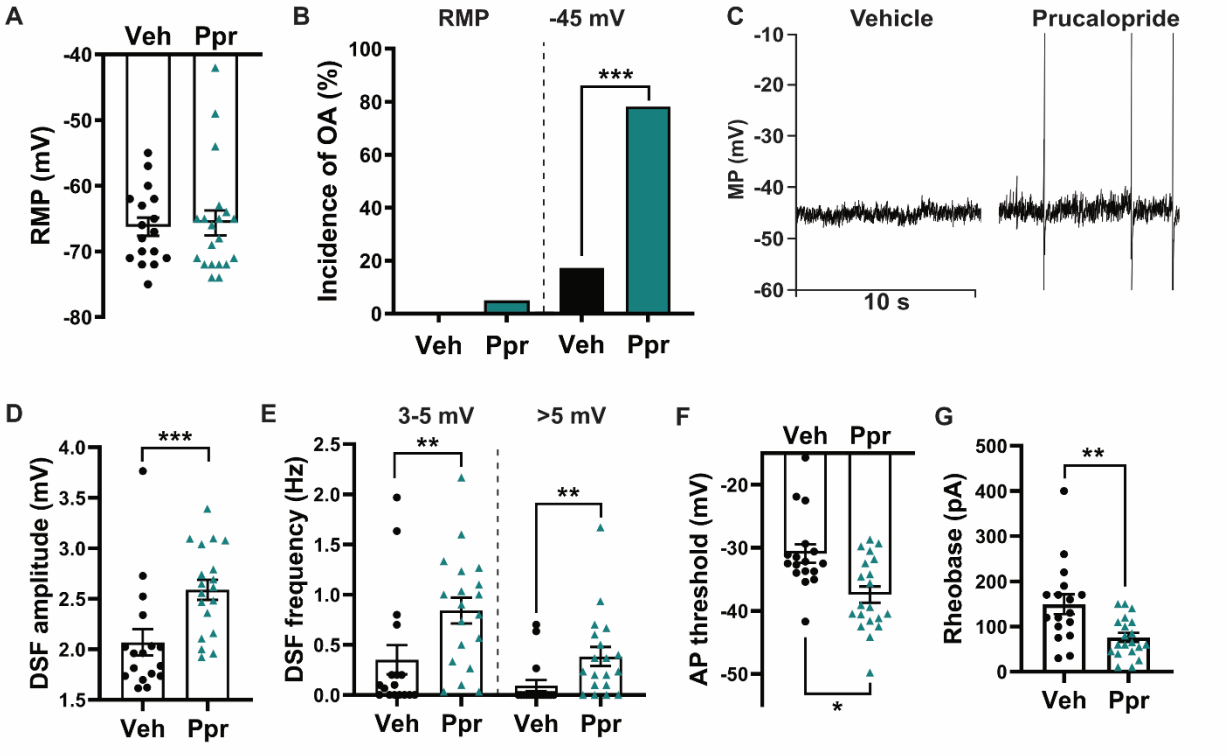


Figure 10. Effects of prucalopride on hyperexcitability of probable nociceptors. (**A**) Lack of effect of prucalopride on RMP measured during electrophysiological recordings from cells continuously exposed to 300 nM prucalopride before and during recording. Mean ± SEM. (**B**) Effect of prucalopride on the incidence of OA determined from ≥60-s recordings at RMP then 30-s recordings at ~-45 mV. *** $p < 0.001$. (**C**) Representative traces of neurons treated with vehicle (DMSO) or prucalopride and depolarized to -45 mV. (**D, E**) Effects of prucalopride on the mean DSF amplitude and the frequency of medium- (3-5 mV) and large-amplitude (> 5 mV) DSFs during 30-s depolarization to -45 mV. Mean ± SEM. ** $p < 0.01$ *** $p < 0.001$. (**F**) Effect of prucalopride on the AP voltage threshold. Mean ± SEM. * $p < 0.05$. (**G**) Effect of prucalopride on the rheobase. Mean ± SEM. ** $p < 0.01$.

DRG neurons comprise several distinct subpopulations, even when excluding large cells as done in our patch clamp experiments. Therefore, I used a high-content microscopy approach to examine 5-HT_4-induced PKA responses in DRG neuron subpopulations, as distinguished by the markers CGRP (for peptidergic nociceptors) and IB4-binding (for non-peptidergic nociceptors), as well as soma size (Garza Carbajal et al., 2020). The strongest PKA activation by prucalopride occurred in the nonpeptidergic nociceptors (IB4, 2.2-fold increase, paired t test, $p = 0.0042$), followed by an apparently weaker but still significant response in neurons positive for both IB4 binding and CGRP (IB-CG, 1.5-fold increase, paired t test, $p = 0.011$); no response was observed in the peptidergic nociceptors (CGRP), small- to medium-sized neurons negative for both markers (N), or large neurons (L, XL) (Figure 11).

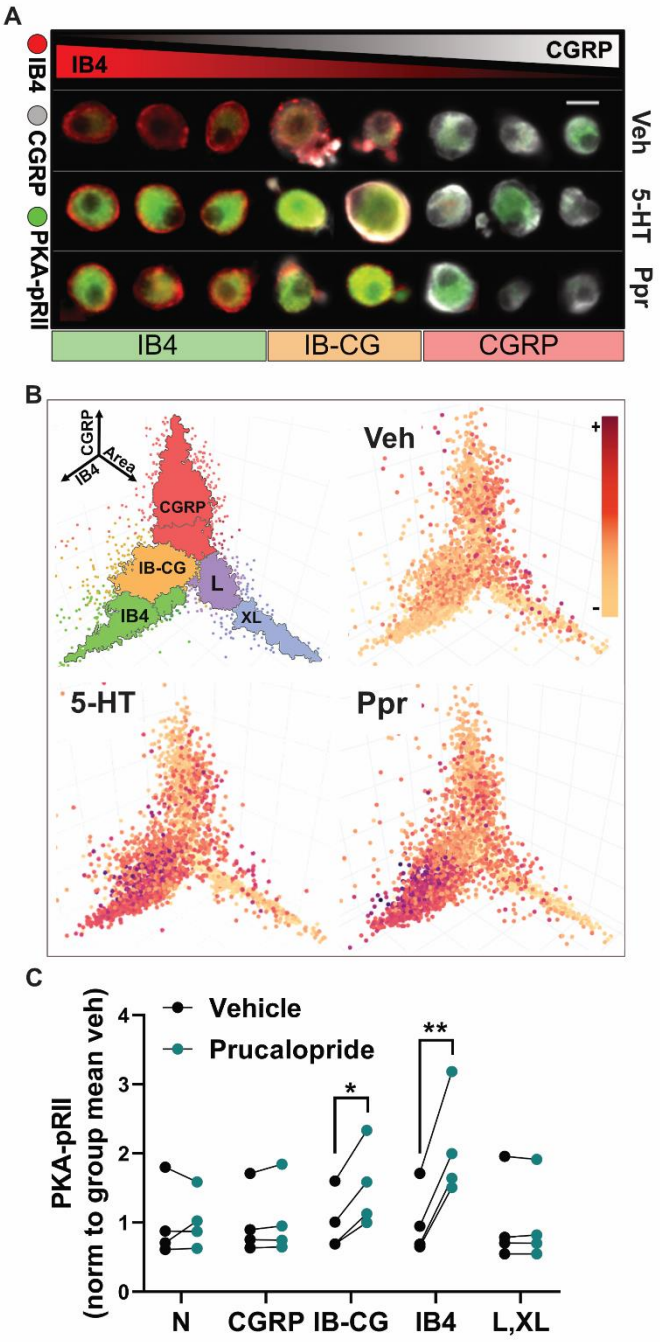


Figure 11. Selective 5-HT$_4$ receptor activation of PKA in DRG neuron subpopulations. (**A**) Examples of cell staining for PKA-pRII. Scale bar = 25 µm. (**B**) PKA-RII phosphorylation in controls, 300 nM 5-HT and 300 nM prucalopride, shown as coordinates of soma area (X-axis), CGRP staining intensity (Y-axis), and IB4 staining intensity (Z-axis). n > 15,000 neurons. (**C**) Prucalopride responses measured by PKA-pRII levels in specific neuronal subpopulations: N (negative for IB4 and CGRP and small somata), CGRP, IB-CG (weak staining for both IB4 and CGRP), L,XL (large and extra-large somata), and IB4. Each point represents the mean from one experiment normalized to the subpopulation baseline from the same experiment (n = 4 rats). * $p < 0.05$ ** $p < 0.01$. Figures in panels A and B made by Anibal Garza Carbajal.

4.3. Conclusions

These results indicate that activation of 5-HT$_4$ receptors is both necessary and sufficient to activate PKA and to promote larger DSFs, reduce AP threshold, and

increase the incidence of OA under depolarized conditions in response to 5-HT, and that this modulation is specific to $IB4^+$ and $IB4^+/CGRP^+$ nociceptors.

Numerous 5-HT receptors are expressed in primary somatosensory neurons and several have been implicated in peripheral sensitization (Loyd et al., 2013). For example, the $5\text{-}HT_{2A}$ antagonist ketanserin can attenuate 5-HT-induced thermal hyperalgesia (Tokunaga et al., 1998), and a $5\text{-}HT_{2B/C}$ antagonist can inhibit 5-HT-induced mechanical allodynia but not 5-HT-induced thermal hyperalgesia in rodents (Lin et al., 2011). It is known from overexpression studies in HEK293T cells that 5-HT can produce robust Ca^{2+} transients via $5\text{-}HT_2$ receptors (Lin et al., 2011). Fast superfusion of 10 µM 5-HT produced rapidly activating and inactivating $[Ca^{2+}]_i$ responses in 67% of capsaicin-sensitive neurons tested in rats (Linhart et al., 2003), in contrast to the lack of effect we found with the $5\text{-}HT_2$ antagonist on effects of 5-60 min bath application of 100-300 nM 5-HT. Similarly, only 3.4% of mouse DRG neurons responded to 1 µM 5-HT with an increase in $[Ca^{2+}]_i$ (Lin et al., 2011). However, significant $[Ca^{2+}]_i$ responses to 5-HT were reported in capsaicin-sensitive rat trigeminal ganglion neurons, which were attenuated by antagonists of $5\text{-}HT_{2A}$ receptors and $5\text{-}HT_3$ receptors (Loyd et al., 2011). Ionotropic $5\text{-}HT_3$ receptors typically activate and desensitize rapidly. In rat DRG neurons, a $5\text{-}HT_2$ antagonist abolished increased AP discharge during depolarizing steps produced by rapid perfusion of 10 µM 5-HT (Salzer et al., 2016).

Different results among these and other studies might be explained by procedural, species, and sex differences, but it seems likely that a major factor contributing to the lack of action potentials or calcium responses evoked by 5-HT at RMP in our experiments arose from our attempt to mimic sustained exposure to 5-HT

likely to occur during *in vivo* inflammation. Slow, prolonged administration of 5-HT is likely to substantially desensitize many 5-HT receptors in nociceptors. In addition, 5-HT_2 and 5-HT_4 receptors may function in partially separate nociceptor subpopulations, as 5-HT_{2A} receptors are mainly expressed in CGRP-synthesizing (peptidergic) small DRG neurons (Okamoto et al., 2002), while we found 5-HT_4-induced PKA-RII phosphorylation (used here as an indirect measurement of cAMP generation) to be largely restricted to $IB4^+$ (nonpeptidergic) and $IB4^+/CGRP^+$ nociceptors. In mice, 5-HT_4 receptor expression was also shown by single-cell RNA-seq profiling to be restricted to nonpeptidergic DRG neurons (Linnarsson Lab, Mousebrain.org; Usoskin et al., 2015).

In addition to the 5-HT_4 family of G_s-coupled receptors, there is evidence for DRG neuron expression of G_s-coupled 5-HT_7 and 5-HT_6 receptors. 5-HT_7 activity can potentiate T-type Ca^{2+} current via PKA-dependent mechanisms in *Xenopus* oocytes co-expressing 5-HT_7 and $Ca_{v3.2}$ (Kim et al., 2006), and a 5-HT_7 agonist mimics potentiating effects of 5-HT on increases of $[Ca^{2+}]_i$ evoked by capsaicin in DRG neurons (Ohta et al., 2006). Functionally, 5-HT_7 is linked to acute and chronic itch but not pain behaviors in mice (Morita et al., 2015). We were unable to block 5-HT activation of PKA in DRG neurons with a selective 5-HT_7 antagonist (SB269970), suggesting 5-HT_7 activity in these neurons is not a major contributor to cAMP signaling evoked by low concentrations of 5-HT.

The evidence for expression of 5-HT_6 receptors in rat lumbar DRG is mixed. For example, 5-HT_6 receptor mRNA was reported to be increased after plantar injection of bee venom (Liu et al., 2005) and was detected above the noise level in my RNA sequencing study of rat DRG (see Chapter 6), but 5-HT_6 receptor mRNA was not

detected in rat DRG neurons by in situ hybridization (Nicholson et al., 2003) or in cultured DRG neurons by RT-PCR (Chen et al., 1998). Because of the close similarity of the effects of the 5-HT_4 receptor agonist prucalopride to those produced by 5-HT, and the complete blockade of 5-HT-induced OA, DSF enhancement, and PKA activation by the 5-HT_4 receptor antagonist GR113808, I conclude that other G_s-coupled 5-HT receptors are less important under conditions used in my experiments, although potential contributions of 5-HT_6 receptors have not been tested directly.

Besides G_s- and G_q-coupled receptors, DRG neurons express G_i-coupled 5-$HT_{1/5}$ receptors. Any 5-$HT_{1/5}$-mediated effects on OA remain to be elucidated and they may be complex because G_i-coupled receptors usually decrease cAMP signaling, and they have been linked to inhibitory effects of 5-HT on nociceptors (Cardenas et al., 1997).

Chapter 5

5-HT potentiates OA in DRG neurons via cAMP-dependent mechanisms

Note: Figures and text are partially reprinted from E. R. Lopez, A. Garza Carbajal, J. B. Tian, A. Bavencoffe, M. X. Zhu, C. W. Dessauer, and E. T. Walters. 2021. Serotonin enhances depolarizing spontaneous fluctuations, excitability, and ongoing activity in isolated rat DRG neurons via 5-HT_4 receptors and cAMP-dependent mechanisms. *Neuropharmacology* 184: 108408. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Elsevier Ltd.

5.1. Introduction

Downstream of 5-HT_4 activation, cAMP production is increased, leading to activation of its effectors, among which PKA is best known (Isensee et al., 2017). The results I presented in Chapter 4 confirmed the activation of PKA by relatively low concentrations of 5-HT (Figure 9). PKA activity has previously been implicated in 5-HT effects on voltage-dependent Na+ current that should reduce AP voltage threshold and rheobase (Cardenas et al., 2001; Gold et al., 1996). Besides 5-HT, other injury- and inflammation-related signals, such as prostaglandins, also bind G_s-coupled receptors, leading to increased cAMP production and pain sensitization (Cui and Nicol, 1995; Ferreira et al., 1978; Hingtgen et al., 1995). In this study, a pharmacological approach was taken to examine the individual roles of the three major cAMP effectors - PKA, EPAC, and HCN - in 5-HT effects on nociceptor hyperexcitability and to examine the effects of general activation of cAMP signaling using a cAMP analog.

5.2. Results

5.2.1. PKA activity is required for potentiation of DSFs and OA by 5-HT

To test the necessity of PKA activity for 5-HT's effects on OA, DSFs, AP voltage threshold, and rheobase, I utilized two different types of PKA inhibitor: H-89, a small-molecule competitive inhibitor, and myr-PKI 14-22, a peptide that mimics endogenous PKA inhibitors. Both H-89 and PKI blocked 5-HT potentiation of OA at -45 mV (Figure 12A; Fisher's exact tests, $p = 0.01$ and $p = 0.0051$, respectively). Similarly, H-89 and PKI both attenuated the frequency of medium-amplitude DSFs (3-5 mV; Kruskal-Wallis, $p = 0.0008$) and large-amplitude DSFs (>5 mV; Kruskal-Wallis, $p = 0.0018$) at -45 mV (Figure 12B). PKI effectively blocked hyperpolarization of the AP voltage threshold by

5-HT (Figure 12C; Kruskal-Wallis, $p = 0.0036$). However, inhibition of PKA activity was not sufficient to significantly block the reduction of rheobase by 5-HT (Figure 12D; Kruskal-Wallis, $p = 0.11$). As anticipated, none of these treatments affected RMP (Figure 12C) or incidence of OA at RMP (Figure 12A). These results demonstrate that PKA plays a critical role in enhancement of DSFs, reduction of AP threshold, and associated potentiation of OA by 5-HT, but plays a lesser role in decreasing rheobase.

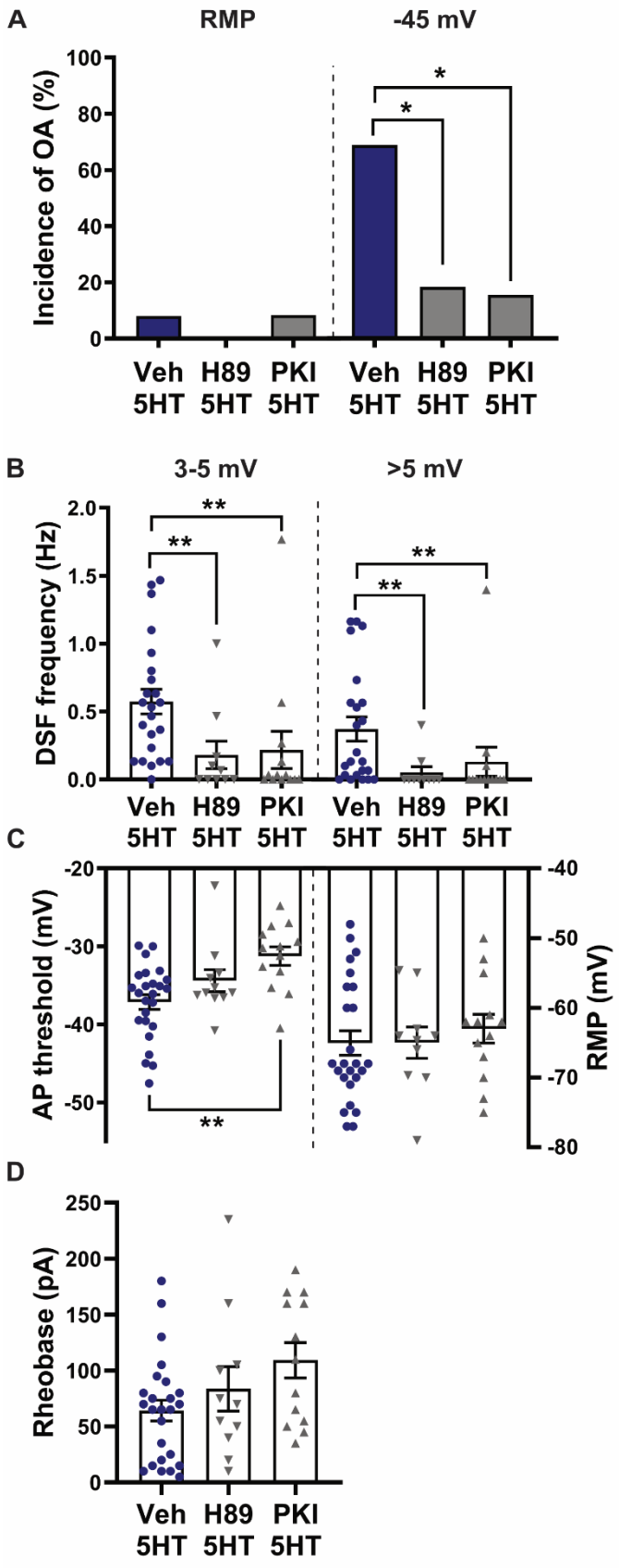


Figure 12. PKA activity is required for potentiation of DSFs and OA by 5-HT. Rat DRG neurons were treated with 10 μM H-89, 1 μM myr-PKI 14-22, or vehicle before and during exposure to 100-300 nM 5-HT. (**A**) Effects of H-89 and PKI on the incidence of OA during 5-HT exposure. The occurrence of OA was determined from ≥ 60-s recordings at RMP then 30-s recordings at ~-45 mV. * $p < 0.025$ (after Bonferroni correction for 2 comparisons). (**B-D**) Effects of H-89 and PKI on the frequency of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs during 30-s depolarization to -45 mV (**B**), AP voltage threshold (**C**), and rheobase (**D**) in the presence of 5-HT. Mean ± SEM. ** $p < 0.01$.

5.2.2. EPAC activity contributes to induction of hyperexcitability by 5-HT

While PKA is the cAMP effector best known for enhancing nociceptor activity and pain, important roles are also played by EPAC (Berkey et al., 2020; Fu et al., 2019; Huang and Gu, 2017; Pan et al., 2019; Singhmar et al., 2018). The relationship between 5-HT signaling and EPAC activity has begun to be elucidated in the brain and spinal cord (Cochet et al., 2013; Fields et al., 2015; Lin et al., 2003), but whether EPAC activity is involved in 5-HT effects on DRG neurons is unknown. To test this possibility, I employed an EPAC1 inhibitor, CE3F4, and EPAC2 inhibitor, ESI05, alone and in combination. Significant attenuation of OA generated at -45 mV in the presence of 5-HT was only observed with combined CE3F4/ESI05 treatment (Figure 13A; Fisher's exact test, $p = 0.002$), although there was a trend toward reduced OA incidence with ESI05 alone ($p = 0.032$, not significant after Bonferroni correction for multiple comparisons). Similarly, the frequency of large-amplitude (>5 mV) DSFs at -45 mV in the presence of 5-HT was significantly attenuated by the combination of CE3F4 and ESI05 (Figure 13B, right panel; Kruskal-Wallis, $p = 0.017$). No significant attenuation of medium-amplitude DSFs (Figure 13B, left; Kruskal-Wallis, $p = 0.24$) or AP threshold (Figure 13C) was observed, but possible trends toward more depolarized AP thresholds in the presence of 5-HT were observed with CE3F4 or ESI05 alone or in combination (1-way ANOVA, $F(3,87) = 2.23$, $p = 0.091$). Weak individual effects of EPAC1 and EPAC2 on AP threshold and DSFs might combine to produce significant effects on OA. No effect of EPAC inhibitors was found on 5-HT-induced reduction of rheobase (Fiure.13D; Kruskal-Wallis, $p = 0.92$), and none of these treatments impacted RMP (Figure 13E; Kruskal-Wallis, $p = 0.89$). Taken together, these findings suggest that EPAC activity contributes

to the increases in DSF amplitude and incidence of OA caused by 5-HT under depolarized conditions.

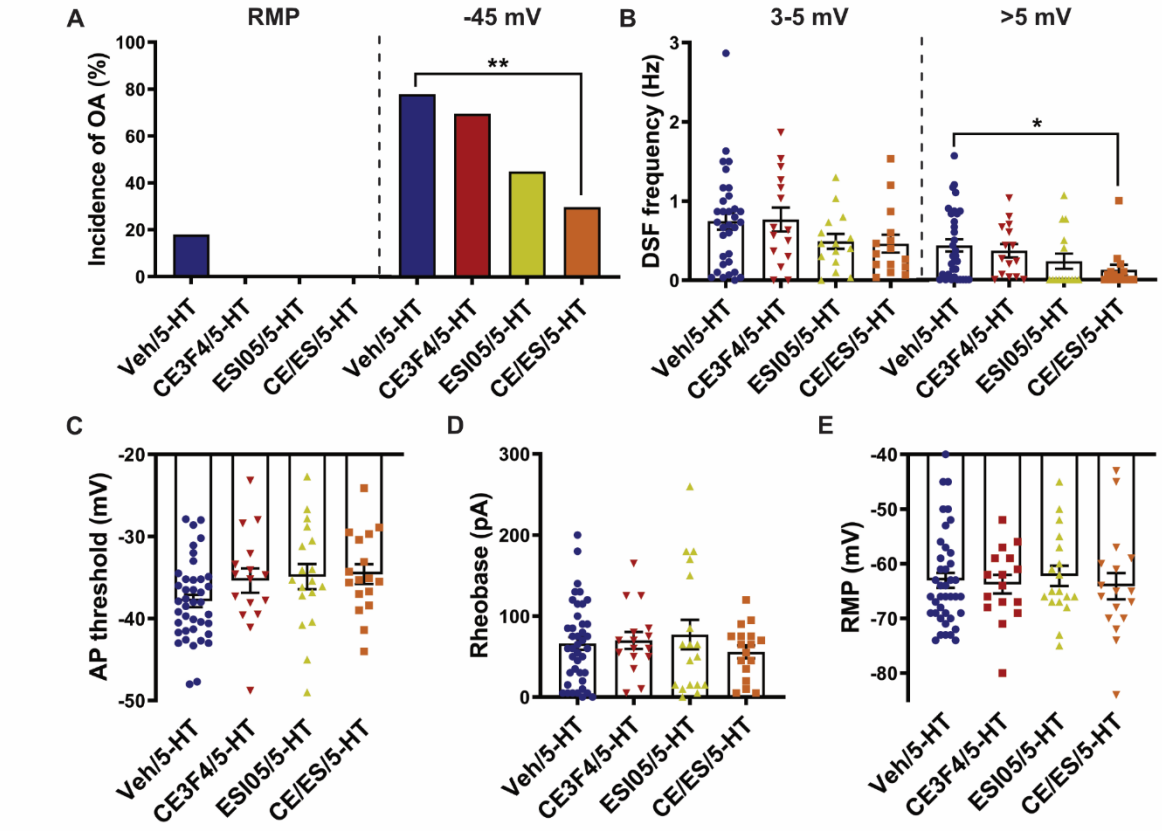


Figure 13. EPAC activity contributes to hyperexcitability induced by 5-HT. Cells were treated with CE3F4 (10 μM) or ESI-05 (5 μM) alone or in combination or vehicle control before and during exposure to 100-300 nM 5-HT. (**A**) Effects of EPAC inhibitors on the incidence of OA was measured during ≥60-sec recordings at RMP then during 30-sec depolarization to -45 mV in the presence of 5-HT. ** $p < 0.0033$ (Bonferroni correction for 3 comparisons). (**B**) Effects of EPAC inhibitors on the frequency of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs during 30-sec depolarization to -45 mV in the presence of 5-HT. Mean ± SEM. * $p < 0.05$. (**C**) Effects of EPAC inhibitors on the AP voltage threshold during exposure to 5-HT. Mean ± SEM. (**D**, **E**) Effects of EPAC inhibitors on rheobase (**D**) and RMP (**E**) during exposure to 5-HT.

5.2.3. Nociceptor hyperexcitability induced by 5-HT does not require HCN activity

The other major cAMP effector known to impact neuronal activity is the HCN channel. HCN channels may contribute modestly to RMP in nociceptors (Du et al., 2014), and they are known to be important for some forms of pain (Emery et al., 2011; Weng et al., 2012). To examine whether HCN plays a role in enhancement of DSFs caused by 5-HT, I tested the effects of blocking this channel with a widely used HCN inhibitor, ZD7288 (e.g., Du et al., 2014), before and during exposure to 5-HT. ZD7288 had no significant effect on the incidence of OA at -45 mV (Figure 14A; Fisher's exact test, $p = 0.26$), frequency of medium- and large-amplitude DSFs at -45 mV (Figure 14B; Mann-Whitney, $p = 0.28$ and $p = 0.32$, respectively), firing frequency at -45 mV (Figure 14C; Mann-Whitney, $p = 0.13$), AP threshold (Figure 14D; t test, $p = 0.64$), or rheobase (Figure 14E; t test, $p = 0.39$), during exposure to 5-HT. These results suggest that HCN activity is not a major contributor to the effects of 5-HT on OA, DSFs, AP threshold, or rheobase in DRG neurons. Unexpectedly, the RMP was more depolarized in the presence of ZD7288 and 5-HT compared to 5-HT alone (mean = -60.9 mV and -67.8 mV, respectively; Mann-Whitney test, $p = 0.0012$; data not shown). This was surprising because ion current through HCN channels in this range of membrane potentials would be expected to be depolarizing, leading to the expectation that blocking the channels would hyperpolarize RMP. Although the significance of this unexpected depolarization of RMP is unknown, it adds to my evidence that 5-HT's effects on HCN signaling do not contribute to the nociceptor hyperexcitability I have investigated.

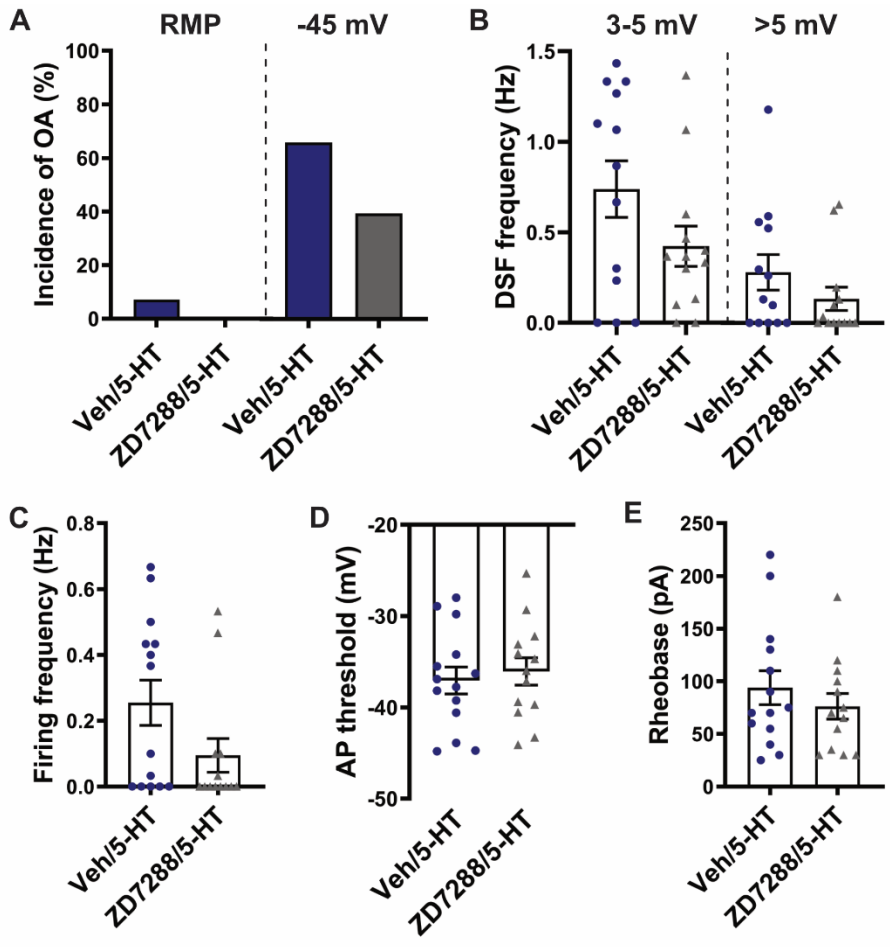


Figure 14. Nociceptor hyperexcitability induced by 5-HT does not require HCN activity. Rat DRG neurons were treated 100 μM ZD7288 before and during exposure to 100 nM 5-HT. (A) Effect of ZD7288 on the incidence of OA during exposure to 5-HT. The occurrence of OA was measured during ≥60-sec recordings at RMP then during 30-sec depolarization to -45 mV. (B-E) Effects of ZD7288 on the frequency of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs during 30-sec depolarization to -45 mV (B), frequency of AP firing during 30-sec recordings at -45 mV (C), AP voltage threshold (D), and rheobase (E) in the presence of 5-HT. Mean ± SEM.

5.2.4. Activation of cAMP signaling is sufficient to induce hyperexcitability in probable nociceptors

While we know that cAMP signaling is important for maintaining chronic nociceptor hyperactivity in vitro after spinal cord injury (Bavencoffe 2016; Berkey 2020),

and that 5-HT and PGE_2 stimulate cAMP signaling in nociceptors, it is not known whether an acute increase in cAMP, which can occur downstream of a number of receptors for various algogenic ligands, is sufficient to promote larger DSFs and OA. To answer this question, I applied the cAMP analog 8-Br-cAMP intracellularly via the patch pipette. Similar to extracellular application of 5-HT, intracellular dialysis of 8-Br-cAMP did not induce OA at rest, but it increased the incidence of OA when the neuron was experimentally depolarized to a holding potential of ~-45 mV (Figure 15A; Fisher's exact test, $p = 0.028$) and reduced the rheobase (Figure 15D; t test, $p = 0.013$). Also similar to 5-HT, 8-Br-cAMP increased the frequency of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs at -45 mV (Figure 15B; Mann-Whitney, $p = 0.0001$ and $p = 0.0024$, respectively) and hyperpolarized the AP voltage threshold (Figure 15C; Mann-Whitney, $p = 0.016$). Unlike 5-HT or the selective $5\text{-}HT_4$ agonist prucalopride, 8-Br-cAMP significantly depolarized the RMP (Figure 15E; t test, $p = 0.0085$), indicating that activation of the cAMP signaling pathway can affect RMP (see also Momin and McNaughton, 2009), albeit not when cAMP signaling is stimulated by the $5\text{-}HT_4$ receptor.

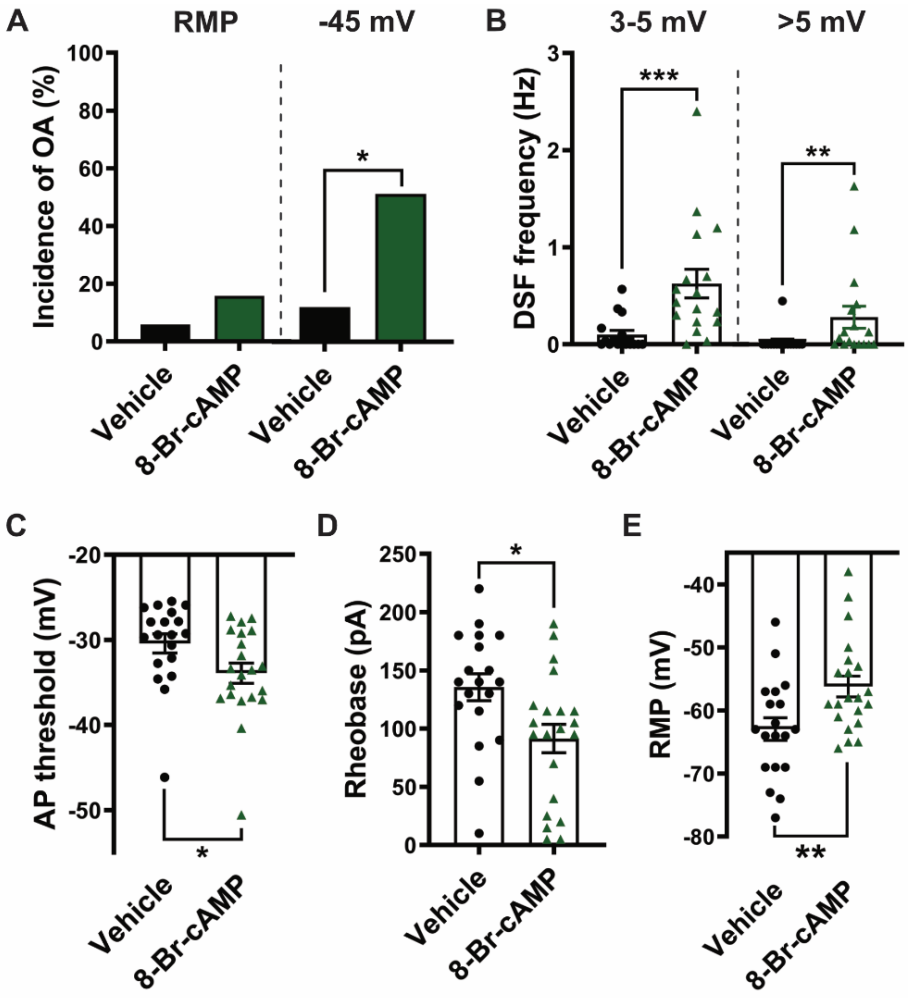


Figure 15. Activation of cAMP signaling by the cAMP analog 8-Br-cAMP is sufficient to potentiate DSFs and OA in isolated DRG neurons. Patch pipettes contained 100 μM 8-Br-cAMP or vehicle in the ICS. (**A**) Effect of 8-Br-cAMP on the incidence of OA. The occurrence of OA was measured during ≥60-sec recording at RMP then during 30-sec depolarization to -45 mV. * $p < 0.05$. (**B-E**) Effect of 8-Br-cAMP on the frequency of medium- (3-5 mV) and large-amplitude (>5 mV) DSFs during 30-sec depolarization to -45 mV (**B**), AP threshold (**C**), rheobase (**D**), and RMP (**E**). Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3. Conclusions

The inhibitors of effectors downstream of cAMP that produced the largest attenuation of 5-HT-induced hyperexcitability were inhibitors of PKA, which blocked enhancement of OA, DSFs, and excitability by 5-HT. PKA activity was previously linked to potentiation by 5-HT of transient receptor potential V1 (TRPV1) function (Ohta et al., 2006) and tetrodotoxin-resistant (TTX-R) Na^+ current (Scroggs, 2011) in isolated DRG neurons, as well as to 5-HT-induced hyperalgesia in vivo (Aley and Levine, 1999). Whether enhanced TRPV1 or TTX-R Na^+ currents are involved in 5-HT-induced OA, and the extent to which the mechanisms described in this study contribute to hyperalgesia in vivo, are not yet known. It is important to note that, while my measure of cAMP production in response to 5-HT and its analogs was type II PKA activity, this does not rule out the interesting possibility of significant contributions of type I PKAs to at least some of the 5-HT effects I observed.

While a number of studies demonstrated a role for EPAC in neuropathic and inflammatory pain (Berkey et al., 2020; Huang and Gu, 2017; Z.-H. Li et al., 2019; Singhmar et al., 2018), the link between 5-HT signaling and EPAC activity in nociceptors had not been explored. I found that EPAC1 and 2 activity (primarily in combination) contributed significantly to the hyperexcitable state induced by 5-HT. The need to block both EPAC isoforms to significantly attenuate the effects of 5-HT in rat DRG neurons is in contrast to the observation that inhibition of either EPAC1 or EPAC2 was sufficient to reverse spinal cord injury-induced nociceptor hyperactivity (Berkey et al., 2020), suggesting differences in the role of EPAC in persistent spinal cord injury-induced OA and acute 5-HT potentiation of OA.

Lastly, I found statistically insignificant trends for reduced excitability when inhibiting HCN channels during 5-HT treatment, suggesting that (at most) HCN channels are a minor contributor to the 5-HT-induced effects I measured. This is consistent with the enhancement of DSFs and OA occurring primarily at relatively depolarized membrane potentials where few HCN channels should be active. Inhibiting any one of the cAMP effectors tested (PKA, EPAC1/2, and HCN) failed to prevent 5-HT's reduction of rheobase (measured from a holding potential of -60 mV), suggesting that additive effects of these components of cAMP signaling, and perhaps other cell signaling pathways, drive this manifestation of 5-HT-induced hyperexcitability.

The promotion of OA by the cAMP analog 8-Br-cAMP showed that activation of cAMP signaling is sufficient to reduce AP threshold and enhance DSFs to increase nociceptor excitability. This suggests that other mediators, such as PGE_2, that activate G_s-coupled receptors on DRG neurons may enhance OA by similar mechanisms. While 8-Br-cAMP depolarized RMP (to ~-57 mV), further depolarization (experimentally, to ~-45 mV) was required to reveal effects on OA, as was also observed with 5-HT. Direct activation of adenylyl cyclase with forskolin produces effects similar to those of 8-Br-cAMP delivery (A. Bavencoffe, E.T. Walters, and C.W. Dessauer, unpublished observations). The lack of a significant increase in OA at RMP induced by 8-Br-cAMP despite the cAMP analog depolarizing RMP, reducing AP threshold, and enhancing DSFs can be explained by its insufficient alteration of some or all of the electrophysiological components that drive OA. Specifically, the hyperpolarization of AP threshold and increased incidence of large DSFs induced by 8-Br-cAMP were modest compared to what was observed with 5-HT, and the depolarization of RMP by 8-Br-cAMP to ~-57 mV is not close to the -45 mV holding potential used to enable OA during

5-HT treatment. The weaker effects of a relatively high concentration of 8-Br-cAMP compared to those of low doses of 5-HT are consistent with findings that OA generation in nociceptors involves interaction of cAMP signaling with at least one other signaling pathway. Interestingly, depolarization to -45 mV, where we see the marked effects on OA and DSFs with cAMP activation, also activates the extracellular signal-regulated kinase (ERK) pathway in $IB4^+$ nociceptors (Garza Carbajal et al., 2020).

Chapter 6

Effects in the DRG of deep tissue incision in a rat model of post-surgical pain

6.1. Introduction

The studies described thus far have provided detailed insight into cell signaling mechanisms that promote OA, in part by enhancing the underlying DSFs. To gain further insight into the contribution of DSFs and OA to different types of pain and the relevance of the mechanisms uncovered in previous chapters, it is important to examine pain and OA in a model that mimics a pain condition seen in humans. One such model is the deep tissue plantar incision rat model of postsurgical pain (Brennan et al., 1995). With 80% of surgical patients experiencing postoperative pain and 86% of these patients reporting the pain as moderate, severe, or extreme, it is clear that postsurgical pain is an important clinical problem for which new treatment options are needed (Apfelbaum et al., 2003; Gan et al., 2014; Gupta et al., 2010). It is estimated that 10-50% of surgical patients develop persistent pain that lasts for months, and 2-10% of patients describe this persistent postoperative pain as severe (Kehlet et al., 2006). There is evidence that more severe acute postsurgical pain increases the likelihood of developing chronic pain (Callesen et al., 1999; Katz et al., 1996; Perkins and Kehlet, 2000; Tasmuth et al., 1996). Thus, managing postsurgical pain early has the potential to prevent persistent postoperative pain.

In the common rat model of incisional pain (Brennan et al., 1995), a deep tissue incision (through skin, fascia, and muscle) on the plantar aspect of the hind paw induces pain and nociceptor ongoing activity (Xu and Brennan, 2010). Guarding behavior, a measure of non-evoked pain, and heat hyperalgesia were demonstrated to last up to five days post-incision in the incised paw (Xu and Brennan, 2010). Peripheral *in vivo* electrophysiological recordings showed ongoing activity in nociceptors one day post-

incision that correlated with guarding pain behavior and was resolved by seven days post-incision (Xu and Brennan, 2010).

Changes in pro-inflammatory cytokines, growth factors, cell signaling, or ion channel activation have been identified that contribute to pain after plantar incision. For example, p38 MAPK phosphorylation increases in the DRG following deep tissue incision and contributes to peripheral nerve sensitization (Mizukoshi et al., 2013), and this was linked to macrophage accumulation and increased TNF-α in the incised plantar tissue (Yamakita et al., 2017). TNF- α is also increased in the DRG and spinal cord post-incision (Chang et al., 2018), and phosphorylation of CREB, a transcription factor phosphorylated by PKA, is enhanced in the lumbar spinal cord following plantar incision (Cui et al., 2010). Upregulation of TRPV1 activity in nociceptor somata was linked to heat hypersensitivity after skin incision (Barabas and Stucky, 2013). A recent study examining the effects of sleep deprivation on post-surgical pain found no significant effect of incision alone on RMP or current threshold in large DRG neurons (which include relatively few nociceptors) 8-9 days post-incision (Q. Li et al., 2019). Electrophysiological changes that occur in nociceptor somata after deep tissue incision have not yet been defined.

While it is known that deep tissue plantar incision leads to OA in nociceptor peripheral fibers in vivo and changes in cell signaling pathways (e.g. p38 MAPK and ERK), inflammation (e.g. increased IL-1β, TNF-α, and IL-6) and ion channel regulation (e.g. TRPV1 activity) in the DRG, whether OA occurs in isolated nociceptor somata and what specific electrophysiological alterations contribute to OA after deep tissue plantar incision have not been explored.

6.2. Results

6.2.1. OA persists in isolated nociceptors after deep tissue incision

To determine whether OA persists in isolated nociceptors after deep tissue incision, how long it persists, and what neurophysiological alterations contribute, I used whole-cell patch clamp recording of dissociated DRG neurons. Neurons were dissociated from the L4-L6 DRG as these contain the somata of sensory neurons that innervate the incised area of the hind paw. Neurons were dissociated on postoperative days (POD) 1, 7, and 14, and ipsilateral and contralateral sides were kept separate. Based on the correlation between OA in isolated DRG neurons and OA and/or behavioral alterations *in vivo* in other pain models, as well as the brief time course of electrophysiological and behavioral alterations previously described for the incision model (lasting less than 1 week), I expected to observe OA in DRG neurons isolated from the side ipsilateral to the incision on POD1 but not on POD7. Surprisingly, the largest increase in OA at rest (often referred to as spontaneous activity) occurred in the probable nociceptors isolated from the ipsilateral side on POD7 (Figure 16A, C; Fisher's exact test, $p = 0.0012$). Ongoing activity during artificial depolarization to -45 mV was significantly increased in ipsilateral neurons isolated on POD1 and POD7 compared to those isolated from naïve, uninjured rats (Figure 16C; Fisher's exact test, $p = 0.0031$ and $p = 0.0003$, respectively).

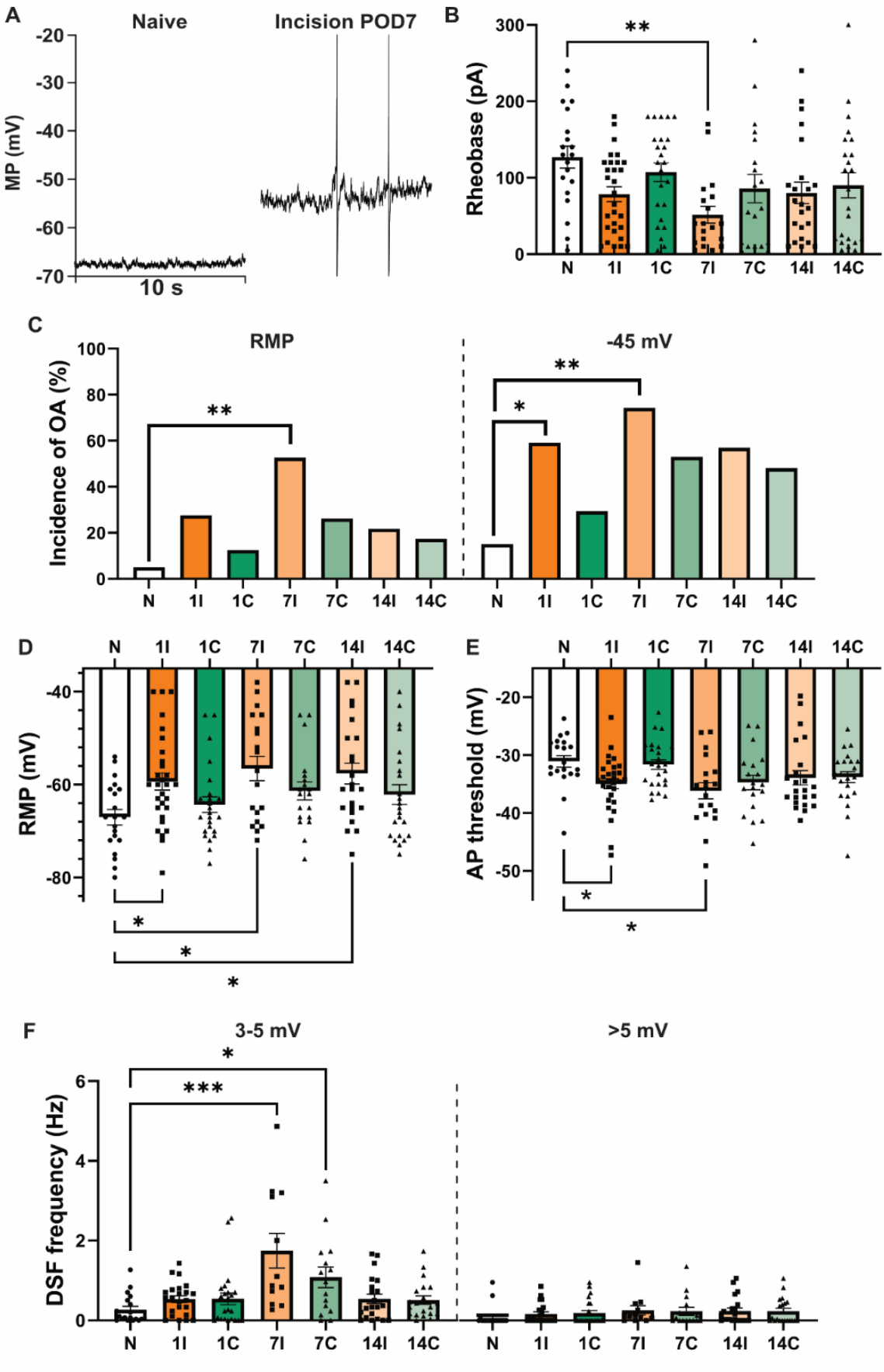

A
Naive
Incision POD7
MP (mV)
-20
-30
-40
-50
-60
-70
10 s
B
Rheobase (pA)
300
200
100
0
**
N
1I
1C
7I
7C
14I
14C
C
RMP
-45 mV
Incidence of OA (%)
100
80
60
40
20
0
**
*
**
N
1I
1C
7I
7C
14I
14C
D
RMP (mV)
-40
-60
-80
*
*
*
E
AP threshold (mV)
-20
-30
-40
-50
*
*
F
3-5 mV
>5 mV
DSF frequency (Hz)
6
4
2
0
*
***
N
1I
1C
7I
7C
14I
14C

Figure 16. Hyperactivity persists in isolated DRG neurons after deep tissue incision. (**A**) Representative traces of naive and post-incision (isolated on postoperative day 7) neurons at rest. (**B**) Effect of incision on the rheobase. Mean ± SEM. ** $p < 0.01$. (**C**) Effect of incision on the incidence of OA determined from ≥60-s recordings at RMP then 30-s recordings at ~-45 mV. * $p < 0.0083$ ** $p < 0.0017$ (Bonferroni corrections for 6 comparisons). (**D**) Effect of incision on RMP measured during ≥60-s recordings. Mean ± SEM. * $p < 0.05$. (**E**) Effect of incision on the AP voltage threshold. Mean ± SEM. * $p < 0.05$. (**F**) Effects of incision on the frequency of medium- (3-5 mV) and large-amplitude (> 5 mV) DSFs during 30-s depolarization to -45 mV. Mean ± SEM. * $p < 0.05$ *** $p < 0.001$. DSF data processed by Michael Zhu.

Deep tissue incision was associated with depolarization of the RMP (a hyperexcitable effect) in probable nociceptors isolated from the ipsilateral side on POD1, 7, and 14 (Figure 16D; Kruskal-Wallis, $p = 0.0443$, $p = 0.0234$, and $p = 0.0207$, respectively). The AP threshold was significantly hyperpolarized in ipsilateral neurons isolated on POD1 and 7 (Figure 16E; one-way ANOVA, $p = 0.0453$ and $p = 0.0106$, respectively). The frequency of medium-amplitude (3-5 mV) DSFs measured during depolarization to -45 mV was markedly increased in ipsilateral DRG neurons isolated on POD7 (Figure 16F; Kruskal-Wallis, $p = 0.0003$). Interestingly, DRG neurons isolated from the contralateral side at this timepoint also exhibited a significantly increased frequency of medium-amplitude DSFs (Figure 16F; Kruskal-Wallis, $p = 0.0142$). There was no statistically significant effect on large-amplitude (> 5 mV) DSFs (Figure 16F; Kruskal-Wallis, $p = 0.1234$). Additionally, the rheobase was significantly reduced in ipsilateral DRG neurons isolated on POD7 (Figure 16B; Kruskal-Wallis, $p = 0.0026$).

To determine whether this unexpected persistence of nociceptor hyperactivity was associated with more prolonged pain behavior than what has been shown

previously, I examined guarding behavior at 1, 7, and 14 days post-incision. A previous study (Xu and Brennan, 2010) correlated guarding behavior, a measure of non-evoked pain with *in vivo* spontaneous activity (equivalent to OA) in C-fiber nociceptors. They observed increased guarding behavior on POD1 but not on POD6 in rats with deep tissue incision, and guarding had resolved by POD1 in rats with incision through the skin only. Similarly, I found guarding behavior to be significantly increased on POD1 (Figure 17; Kruskal-Wallis, $p = 0.0035$) but absent on POD7. The apparent increase in guarding behavior in sham incised rats on POD1 was not statistically significant (Figure 17; Kruskal-Wallis, $p = 0.1851$). This suggests that our deep tissue incision condition is comparable in the time course of non-evoked pain resolution to published studies.

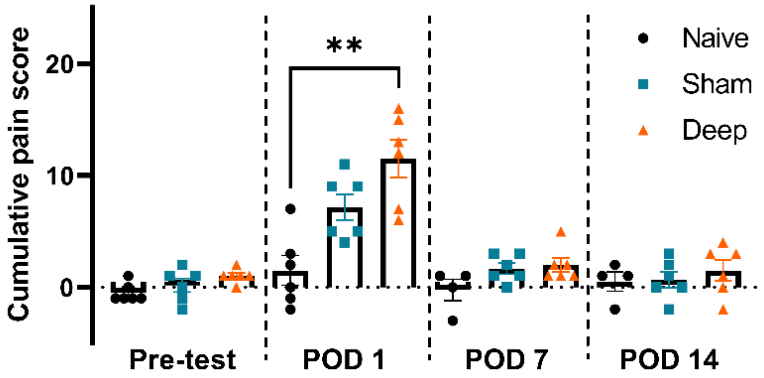
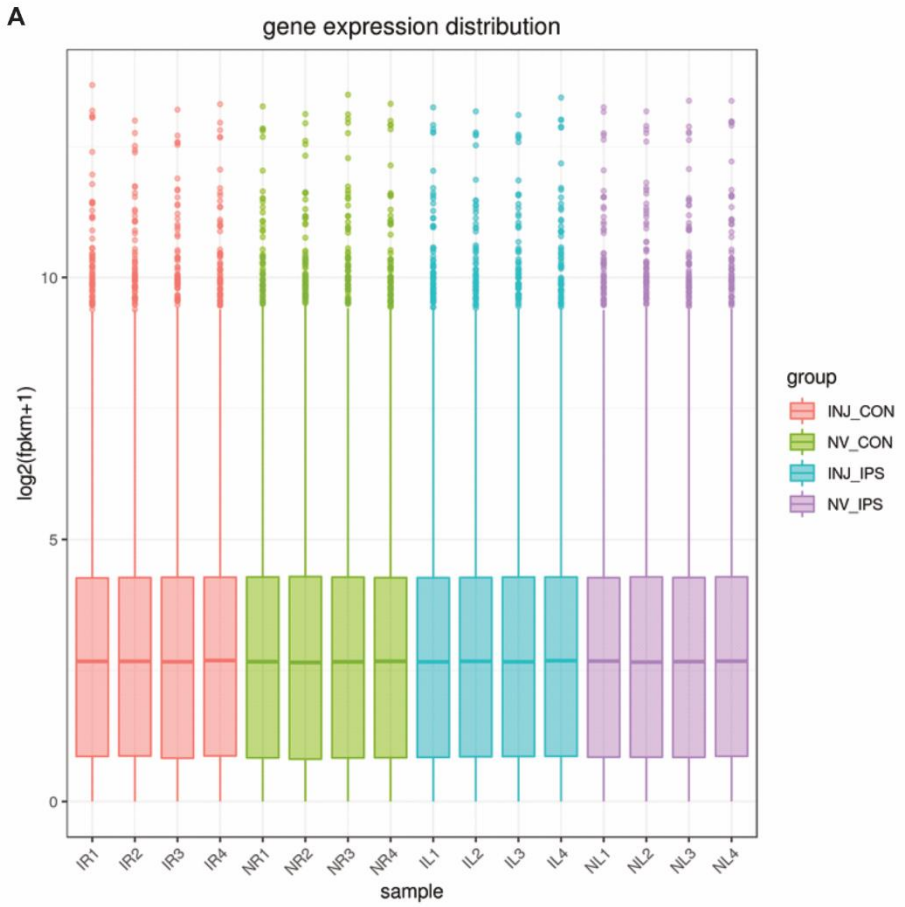


Figure 17. Guarding pain behavior is resolved by POD7. Guarding behavior measured during 12 one-minute observation periods every 3 or 4 minutes. Mean ± SEM. 2-way ANOVA, $F(11,49) = 13.56$, $p < 0.0001$, followed by separate Kruskal-Wallis tests for each time point, ** $p < 0.01$. POD, postoperative day; Deep, incision through skin, fascia, and muscle (deep tissue incision); Sham, incision through skin and possibly fascia but not muscle; Naïve, no incision.

6.2.2. Global transcriptome analysis of rat DRG to identify genes involved in persistent nociceptor hyperexcitability after deep tissue incision

The unexpected findings of hyperexcitability persisting in isolated nociceptors for at least 7 to 14 days post-incision (Figure 16D) and of hyperexcitable effects in DRG neurons contralateral to the incision (Figure 16F) raise many questions, including whether alterations in gene expression might underlie this persistent hyperexcitability. To address the question of whether gene expression changes accompany hyperactivity of DRG neurons at 7 days post-incision, the timepoint where the greatest effect on OA at RMP was observed (Figure 16C), I turned to the unbiased approach of RNA sequencing. To detect potential changes in gene expression bilaterally, RNA was extracted separately from ipsilateral L4-6 DRG and contralateral L4-6 DRG and similarly from left (corresponding to the ipsilateral side of incised rats) and right (corresponding to the contralateral side) L4-6 DRG from naïve rats. Therefore, ipsilateral and contralateral sides each had their own control in case lateralized differences occur normally. All samples had similar levels of gene expression (Figure 18A), and replicates correlated well with each other (Figure 18B). For differential gene expression, cutoffs were set at 2-fold change and p value < 0.01.


A
gene expression distribution
log2(fpkm+1)
10
5
0
IR1
IR2
IR3
IR4
NR1
NR2
NR3
NR4
IL1
IL2
IL3
IL4
NL1
NL2
NL3
NL4
sample
group
INJ_CON
NV_CON
INJ_IPS
NV_IPS

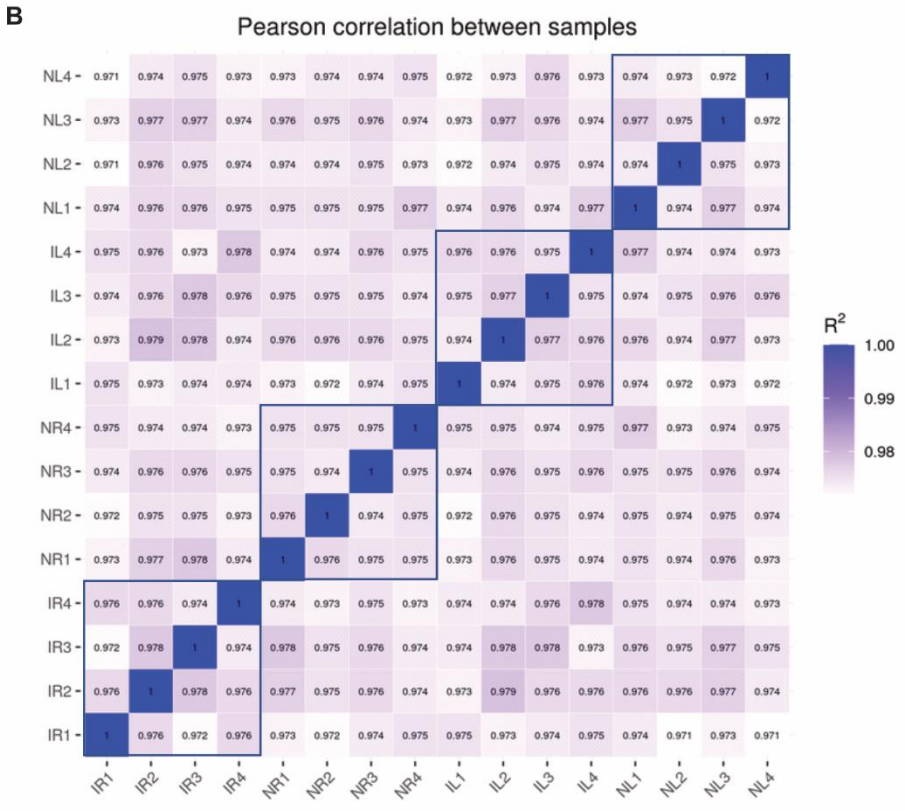

B
Pearson correlation between samples
NL4 0.971 0.974 0.975 0.973 0.973 0.974 0.974 0.975 0.972 0.973 0.976 0.973 0.974 0.973 0.972 1
NL3 0.973 0.977 0.977 0.974 0.976 0.975 0.976 0.974 0.973 0.977 0.976 0.974 0.977 0.975 1 0.972
NL2 0.971 0.976 0.975 0.974 0.974 0.974 0.975 0.973 0.972 0.974 0.975 0.974 0.974 1 0.975 0.973
NL1 0.974 0.976 0.976 0.975 0.975 0.975 0.975 0.977 0.974 0.976 0.974 0.977 1 0.974 0.977 0.974
IL4 0.975 0.976 0.973 0.978 0.974 0.974 0.976 0.975 0.976 0.976 0.975 1 0.977 0.974 0.974 0.973
IL3 0.974 0.976 0.978 0.976 0.975 0.975 0.975 0.974 0.975 0.977 1 0.975 0.974 0.975 0.976 0.976
IL2 0.973 0.979 0.978 0.974 0.976 0.976 0.976 0.975 0.974 1 0.977 0.976 0.976 0.974 0.977 0.973
IL1 0.975 0.973 0.974 0.974 0.973 0.972 0.974 0.975 1 0.974 0.975 0.976 0.974 0.972 0.973 0.972
NR4 0.975 0.974 0.974 0.973 0.975 0.975 0.975 1 0.975 0.975 0.974 0.975 0.977 0.973 0.974 0.975
NR3 0.974 0.976 0.976 0.975 0.975 0.974 1 0.975 0.974 0.976 0.975 0.976 0.975 0.975 0.976 0.974
NR2 0.972 0.975 0.975 0.973 0.976 1 0.974 0.975 0.972 0.976 0.975 0.974 0.975 0.974 0.975 0.974
NR1 0.973 0.977 0.978 0.974 1 0.976 0.975 0.975 0.973 0.976 0.975 0.974 0.975 0.974 0.976 0.973
IR4 0.976 0.976 0.974 1 0.974 0.973 0.975 0.973 0.974 0.974 0.976 0.978 0.975 0.974 0.974 0.973
IR3 0.972 0.978 1 0.974 0.978 0.975 0.976 0.974 0.974 0.978 0.978 0.973 0.976 0.975 0.977 0.975
IR2 0.976 1 0.978 0.976 0.977 0.975 0.976 0.974 0.973 0.979 0.976 0.976 0.976 0.976 0.977 0.974
IR1 1 0.976 0.972 0.976 0.973 0.972 0.974 0.975 0.975 0.973 0.974 0.975 0.974 0.971 0.973 0.971
IR1 IR2 IR3 IR4 NR1 NR2 NR3 NR4 IL1 IL2 IL3 IL4 NL1 NL2 NL3 NL4
R2
1.00
0.99
0.98

Figure 18. Gene expression distribution and correlation between samples. (A) Box plots displaying the distribution of gene expression levels and FPKM among different samples. FPKM, number of fragments per kilobase length of gene per million fragments. (B) Correlation coefficient matrix. R^2, square of Pearson correlation coefficient (R). INJ_CON, incised (injured) contralateral group; NV_CON, naïve contralateral group; INJ_IPS, incised ipsilateral group; NV_IPS, naïve ipsilateral group; IR, incised contralateral sample; NR, naïve contralateral sample; IL, incised ipsilateral sample; NL, naïve ipsilateral sample.

In ipsilateral DRG, 6 genes, *Vip*, *Fam131a*, *RragB*, *Stac2*, *Ecel1*, and *Yif1b* were significantly upregulated compared with naïve controls, and 1 gene, *Zfp622*, was downregulated (Figure 19; Table 1). In contralateral DRG, only one gene was differentially expressed; *AC115341.1* was downregulated compared to naïve controls (Figure 20; Table 2). The differentially expressed genes are discussed in section 6.3.

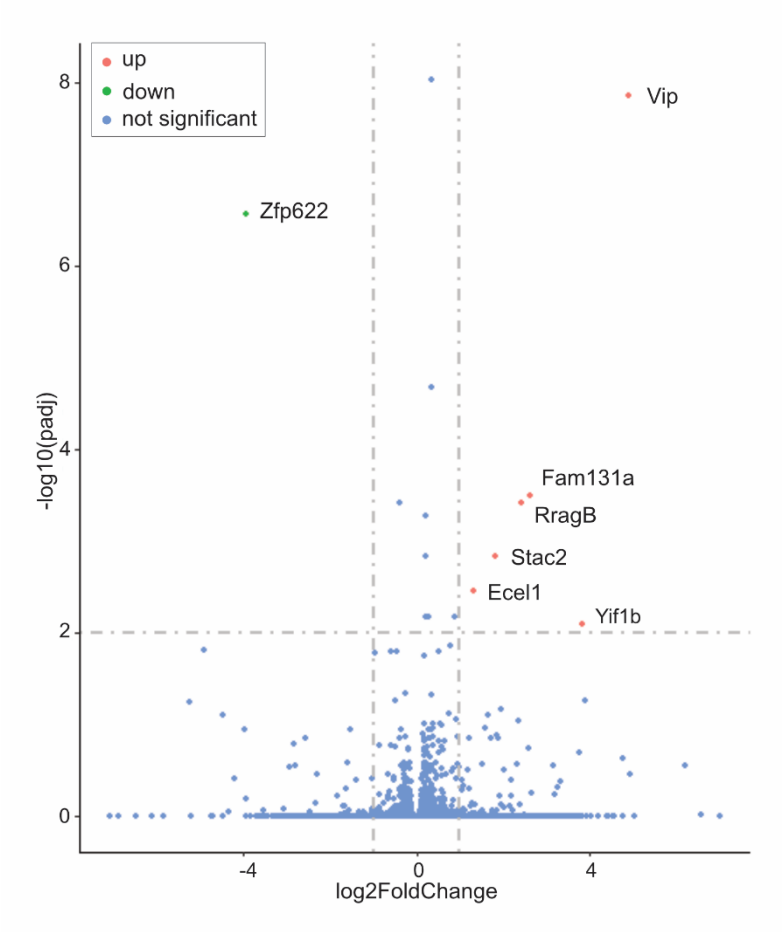


Figure 19. Volcano plot of ipsilateral differentially expressed genes 7 days post-incision. Expression in L4-6 DRG from the ipsilateral side of incised rats compared to L4-6 DRG from the same side of naïve rats. Points represent genes labeled with the gene name. Dashed lines represent cutoffs of 2-fold change and p value < 0.01.

Table 1. Genes differentially expressed in ipsilateral DRG at 7 days post-incision compared to naïve controls.

Gene Name	Description	Log2 Fold Change	padj	Chromosome
Vip	Vasoactive intestinal peptide	4.8922	0.0000	1
Zfp622	Zinc finger protein	-3.9261	0.0000	2
Fam131a	Family w/ sequence similarity 131, member A	2.6137	0.0003	11
RragB	Ras-related GTP binding B	2.4283	0.0004	X
Stac2	SH3 & cysteine rich domain 2	1.8303	0.0015	10
Ecel1	Endothelin converting enzyme-like 1	1.3285	0.0036	9
Yif1b	Yip1 interacting factor homolog B	3.8085	0.0081	1

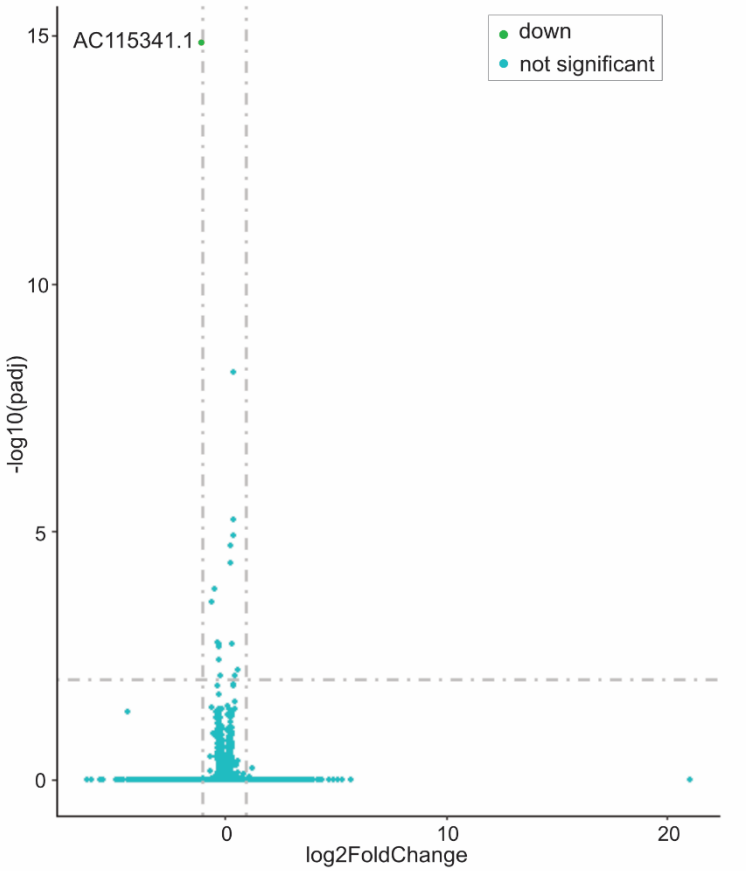


Figure 20. Volcano plot of contralateral differentially expressed genes 7 days post-incision. Expression in L4-6 DRG from the contralateral side of incised rats compared to L4-6 DRG from the same side of naïve rats. Points represent genes labeled with the gene name. Dashed lines represent cutoffs of 2-fold change and $p < 0.01$.

Table 2. Genes differentially expressed in contralateral DRG at 7 days post-incision compared to naïve controls.

Gene Name	Description	Log2 Fold Change	padj	Chromosome
AC115341.1	Serine-threonine protein phosphatase 2A activator	-1.0558	0.0000	3

To establish the noise threshold, I used an approach that takes into account sample-to-sample variation, as described recently by Koch, et al. (Koch et al., 2018). Variability between samples was visualized for this purpose by comparing the number of genes expressed at different cutoffs across all samples. The samples did not become indistinguishable until an FPKM cutoff of 0.5 was reached, permitting a low counts threshold of FPKM = 0.5 (Figure 21). All differentially expressed genes were above this determined noise level in at least 3 out of 4 replicates in at least one condition (incised or naïve) (Table 3).

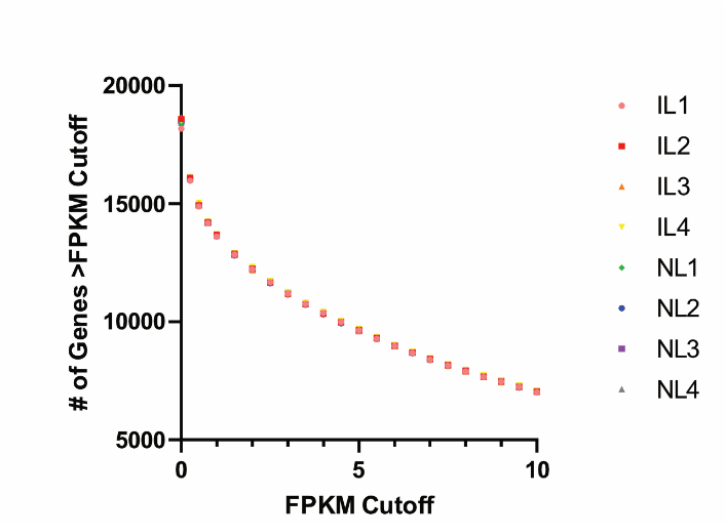


Figure 21. Quantification of genes with expression above different FPKM cutoff values. The gene expression distributions across different samples align quickly, leading to a threshold of FPKM = 0.5. IL, incised ipsilateral; NL, naïve ipsilateral; 1-4, biological replicates.

Table 3. FPKM values of genes differentially expressed in ipsilateral and contralateral DRG at 7 days post-incision. IL, incised ipsilateral; NL, naïve ipsilateral; IR, incised contralateral; NR, naïve contralateral; 1-4, biological replicates.

gene_id	IL1	IL2	IL3	IL4	NL1	NL2	NL3	NL4	gene_name
ENSRNOG00000018808	2.28268558	3.83441997	0.91657168	1.35470858	0.07987836	0.04286206	0.03690832	0.12633287	Vip
ENSRNOG00000059443	0.20308313	0.07354765	0.03832587	0.13704748	2.58854943	1.65783152	1.00314401	1.27661141	Zfp622
ENSRNOG00000045653	1.85310546	0.27719848	0.74505199	0.94243543	0.11594747	0.19553739	0.16837634	0.13971697	Fam131a
ENSRNOG00000050535	0.62671753	0.92122763	1.32188818	0.24878267	0.16673874	0.16267385	0.12606993	0.12785855	RragB
ENSRNOG00000004805	1.63573239	2.42106928	0.80529289	1.59978041	0.52635389	0.60671673	0.16213686	0.53442082	Stac2
ENSRNOG00000019447	0.68393225	0.68922364	0.45642772	0.55295734	0.22823011	0.32366111	0.13558529	0.26642351	Ecel1
ENSRNOG00000020616	11.7440057	13.6040629	1.19461767	21.6211559	0.95890612	0.44103515	0.37977333	1.66100844	Yif1b
gene_id	IR1	IR2	IR3	IR4	NR1	NR2	NR3	NR4	gene_name
ENSRNOG00000022490	36.27219	29.65835	34.10362	36.57926	62.69084	65.93988	84.64073	70.25088	AC115341.1

6.3. Conclusions

With the plantar incision model of post-surgical pain, I have shown thus far that deep tissue incision potentiates OA, depolarizes RMP, and hyperpolarizes AP threshold in DRG neurons isolated from the ipsilateral side 1 day post-incision. Unexpectedly, the largest changes in ipsilateral DRG neurons compared with naïve controls were observed in probable nociceptors isolated 7 days post-incision, with an increased incidence of OA at RMP and at -45 mV, depolarized RMP, hyperpolarized AP threshold, and enhanced DSFs. Depolarization of RMP was also present in DRG neurons isolated 14 days post-incision. Additionally, enhanced DSFs were detected in DRG neurons isolated from the contralateral side 7 days post-incision. These results suggest that hyperexcitable effects of deep tissue incision on nociceptors persist longer than what has been revealed by *in vivo* behavioral tests and that some effects occur bilaterally. This persistent hyperexcitability observed in isolated nociceptors may be the result of a “primed” state induced by the incision that is revealed by the stress of the dissociation procedure. Alternatively, nociceptor hyperexcitability may be actively suppressed *in vivo*, such as by endogenous opioid pathways, and thus is only revealed when the nociceptors are

removed from such suppressive mechanisms, which would be consistent with the recent finding that hyperalgesia can be reinstated by opioid receptor antagonists 21 days post-incision (Custodio-Patsey et al., 2020). Hypotheses and future directions regarding the mechanisms underlying the hyperexcitability in isolated nociceptors lasting longer than *in vivo* pain will be discussed in Chapter 7.

In contrast to a recent study that found 126 genes differentially expressed in DRG 1 day post-incision compared with controls (Tran et al., 2020), my RNA sequencing revealed few transcriptional changes in DRG 7 days post-incision compared to naïve controls, suggesting that alterations at the level of gene expression may not account for the observed effects on nociceptor excitability at this time point. Given the ability of 5-HT to produce covert hyperexcitability (revealed only when membrane potential is depolarized) shown in Chapter 3, an important question was whether the mRNAs for any 5-HT receptors changed in expression after incision. Several 5-HT receptor mRNAs were present in my DRG samples– in descending order of abundance, *Htr3b, Htr3a, Htr1d, Htr1b, Htr4, Htr1a, Htr2a, Htr5b,* and *Htr2c*. However, there was no significant differential expression of any 5-HT receptors 7 days post-injury. The only differentially expressed genes identified are described briefly below.

Vip: The upregulated gene showing the most statistical significance (Figure 19, Table 1) on POD7 in ipsilateral DRG, albeit still at a low level of expression (Table 3), was *Vip*, which encodes vasoactive intestinal peptide (VIP). VIP is a neuropeptide that was originally reported as a vasodilator (Said and Mutt, 1970) and has since been found to have a wide array of physiological and pathological effects, including but not limited to gastrointestinal function and diseases (Iwasaki et al., 2019), cancer (Siddappa and Vege, 2019), and pain (Bourlev et al., 2018; McDougall et al., 2006). VIP carries out its

activities by binding the G_s-coupled receptors VPAC1/2 (Moody and Jensen, 2013) and is widely distributed in the CNS and PNS (Lorén et al., 1979; Said, 1984).

Fam131a: This gene encodes a FAM131 family member. Little is known about the function of these proteins other than their ability to be myristoylated (Suzuki et al., 2010), suggesting a putative role in cell signaling.

RragB: RAS-related GTP-binding protein B (RRAGB), as the name suggests, is a GTPase that is homologous to RAS, a GTPase that is well-known for its role in cancer. GTPases are cellular switches that alternate between an active GTP-bound state and an inactive GDP-bound state. RRAG family GTPases are known to be involved in cellular responses to amino acid availability by regulating signaling through mammalian target of rapamycin complex 1 (mTORC1) (Takahara et al., 2020). The function of RRAGB in the DRG has not been studied. Interestingly, the activity of its homolog, RAS, appears to be involved in nociceptor hyperexcitability stimulated by membrane depolarization (Garza Carbajal et al., 2020).

Stac2: Another gene of interest that was significantly upregulated was *Stac2*, which encodes SH3 and cysteine-rich domain-containing protein 2 (STAC2). About a decade ago, STAC1 and STAC2, of unknown function at the time, were shown to be expressed in distinct subsets of DRG neurons (Legha et al., 2010). Since then, studies in neurons of the brain have uncovered a role for STAC2 in the regulation of voltage-gated Ca^{2+} channel activity (Campiglio et al., 2018; Polster et al., 2018, 2015).

Ecel1: Endothelin converting enzyme like 1 (ECEL1), also known as damage-induced neuronal endopeptidase (DINE) is a neuron-specific membrane-bound

metalloprotease that shares homology with neprilysin and endothelin-converting enzyme, which degrade or process neuropeptides (Benoit et al., 2004; Kiryu-Seo et al., 2000). Transcriptional responses of this gene have been observed with various types of nerve injuries (Kaneko et al., 2017; Kiryu-Seo, 2006). For example, *Ecel1* expression was observed in small sized DRG neurons after sciatic nerve injury. The function and substrate(s) of ECEL1 are largely unknown, but some evidence suggests it has neuroprotective effects (Kaneko et al., 2017; Matsumoto et al., 2016).

Yif1b: YIP1 interacting factor homology B (YIF1B) is involved in anterograde trafficking from the endoplasmic reticulum to the plasma membrane and in the organization of Golgi architecture (Alterio et al., 2015; Matern et al., 2000). YIF1B plays a key role in targeting the 5-HT_{1A} receptor to neuronal dendrites in the brain (Carrel et al., 2008). It is known that YIF1B does not interact with the 5-HT_{1B} receptor (Carrel et al., 2008), but whether or not YIF1B has affinity for other 5-HT receptors, or receptors for other neuromodulators, and what its function is in the DRG are not known.

Zfp622: The only gene found to be downregulated in ipsilateral DRG 7 days post-incision encodes zinc finger protein 622 (ZFP622), also known as zinc finger-like protein 9 (ZPR9), which was originally identified as a substrate of murine protein serine/threonine kinase 38 (MPK38) (Seong et al., 2002). ZNF622 regulates apoptosis signal-regulating kinase 1 (ASK1), transforming growth factor β (TGF-β), and p53 signaling pathways (Seong et al., 2017), and a recent study suggests ZFP622 may act as a cellular antiviral protein in humans (Mun and Punga, 2018). The function of ZFP622 in the DRG has not been studied.

AC115341.1: The only gene found to be differentially expressed in contralateral DRG 7 days post-incision was a pseudogene of *Ptpa*, a gene encoding a serine/threonine protein phosphatase 2A activator. PTPAs accelerate protein folding and catalyze cis-trans isomerization of proline imidic peptide bonds in oligopeptides (www.uniprot.org).

It is important to note that differential expression studies were conducted from RNA extracted from whole DRG tissue, so while predictions can be made based on what is known about the expression of these genes, it is uncertain whether the genes of interest were expressed in nociceptors, other sensory neurons, or non-neuronal cell types. Furthermore, a relatively low expression in the whole DRG does not mean that a given gene could not be expressed to a much greater degree in a subpopulation of DRG neurons of a subset of nociceptors. Finally, increasing evidence indicates that translational modifications of DRG neurons are important for driving pain, perhaps even more important than transcriptional alterations (Megat et al., 2019). Predictions and future directions regarding the potential roles of the differentially expressed genes detected in this study are discussed in Chapter 7.

Chapter 7

Concluding remarks and future directions

7.1. Summary of conclusions

The major findings of this study are (1) that low concentrations of 5-HT promote acute OA through the G_s-coupled 5-HT_4 receptor and downstream cAMP signaling, predominantly via activation of PKA, with some contributions from EPAC (Figure 22), and (2) that OA persists in isolated nociceptors for weeks after deep tissue plantar incision, with effects on RMP, AP threshold, and DSFs but with few transcriptional changes associated with this persistent OA.

7.2. Functional implications of 5-HT_4-dependent cAMP signaling in nociceptors

Nociceptor OA induced by injury or inflammation may be generated in peripheral terminals, axonal neuromas, and/or the soma, with each site potentially exposed persistently to inflammatory mediators, including 5-HT (discussed by Walters, 2019). Our findings set the stage for multidisciplinary approaches to define potentially important contributions of 5-HT-stimulated cAMP signaling in nociceptors – within the DRG and at sites of injury – to behaviorally expressed ongoing pain.

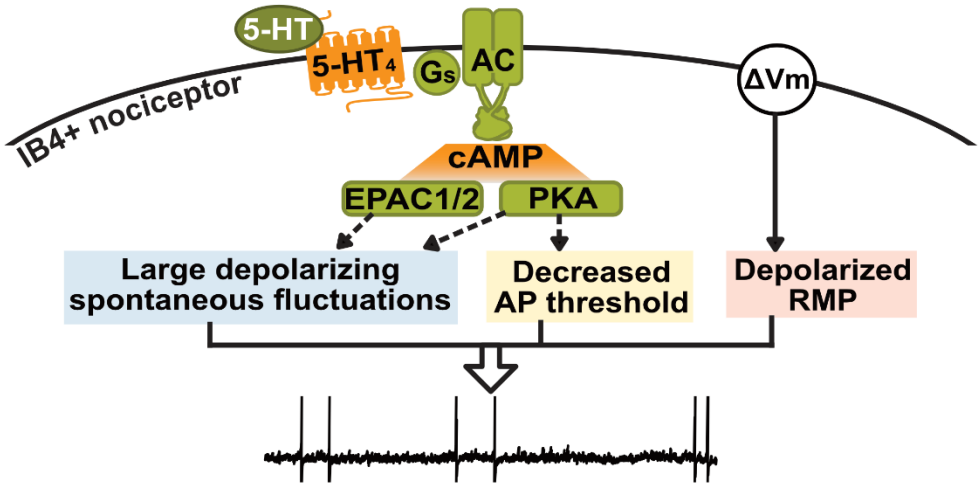


Figure 22. Graphical summary of 5-HT$_4$-mediated potentiation of OA in nociceptors. In isolated nociceptors exposed to 5-HT for 5-60 min, the G_s-coupled 5-HT$_4$ receptor stimulates cAMP signaling, with the effectors PKA and EPAC causing hyperpolarized AP threshold and enhanced DSFs, thereby potentiating OA in the presence of a depolarizing stimulus. reprinted from E. R. Lopez, A. Garza Carbajal, J. B. Tian, A. Bavencoffe, M. X. Zhu, C. W. Dessauer, and E. T. Walters. 2021. Serotonin enhances depolarizing spontaneous fluctuations, excitability, and ongoing activity in isolated rat DRG neurons via 5-HT$_4$ receptors and cAMP-dependent mechanisms. *Neuropharmacology* 184: 108408. Use of the text and figures are granted for use in a dissertation by an author without formal licensing from Elsevier Ltd.

The nociceptor hyperexcitability mechanisms described in Chapters 3-5 may contribute to pain associated with peripheral tissue injury, such as incisional pain. As described in Chapter 1, 5-HT is one of several mediators released by activated mast cells. Mast cells release 5-HT when activated by injuries such as plantar incision (Oliveira et al., 2011). Serotonin-cAMP signaling promotes OA by reducing AP threshold and enhancing DSFs, with the OA generated in the periphery and/or soma likely to contribute to ongoing pain and central sensitization. The effects of 5-HT-cAMP signaling

on rheobase may be particularly relevant in the periphery, where increased 5-HT_4 activity in nociceptors may render their peripheral terminals more sensitive to sensory generator potentials and thereby contribute to hyperalgesia.

As my studies were conducted in isolated DRG neurons, the mechanisms of nociceptor hyperexcitability defined by these studies may be especially relevant in the DRG. Besides mast cells, 5-HT is also stored in platelets and released upon platelet activation. Interestingly, 5-HT is elevated in the plasma as well as in the CSF acutely following spinal cord injury (Brodner et al., 1980; Sharma et al., 1993). A study also found significantly elevated plasma 5-HT levels in patients with primary fibromyalgia syndrome (PFS), a condition characterized by diffuse chronic pain, compared to controls (Legangneux et al., 2001). Because DRG are perfused by blood in addition to CSF (Abram et al., 2006; Godel et al., 2016), the contribution of circulating and intrathecal 5-HT to the early C-fiber OA generated within the DRG in vivo after spinal cord injury (Bedi et al., 2010) or to PFS pain warrants further investigation.

7.3. Advantages and potential pitfalls of a pharmacological approach to studying molecular mechanisms

The necessity and relative contribution of a given pathway or protein to a physiological outcome of interest can be determined experimentally by examining the effects of functionally removing the pathway or protein. Functional removal can be achieved by genetic deletion or suppression or by pharmacological inhibition. Pharmacological approaches to studying molecular mechanisms include antibody-based, peptide-based, and (most commonly) small-molecule based treatments to inhibit,

or in some cases activate, a protein of interest. Compared to genetic approaches, pharmacological inhibition typically takes place on a significantly shorter time scale. On the one hand, creating a knockout mouse when one is not already available can take about a year from start to finish (www.med.umich.edu/tamc), and effective knockdown of a gene may involve a slow turnover rate of already existing gene products. On the other hand, the effects of pharmacologically inhibiting a protein typically occur within seconds to hours, depending on the mode of delivery and the outcome being measured. Pharmacological tools also provide the power to observe the effects of inhibiting the target of interest in real time (avoiding compensatory effects that often occur with transgenic approaches) and the ability to compare functional outcomes before and after inhibition of the target within the same animal or the same cell. Pharmacological studies also provide an additional degree of translatability since, in many cases, it is more feasible to treat a human with a drug than to knock out or knock down a gene in a patient.

Nonetheless, there are a number of major weaknesses to keep in mind when employing a pharmacological approach. Notably, it is common for drugs to exhibit significant off-target effects, especially in the case of kinase inhibitors (Davis et al., 2011; Michel and Seifert, 2015). Cell permeability is also a common issue for drugs with intracellular targets. Different drugs differ in their ability to cross the plasma membrane, and the extracellular concentration of drug is often a poor predictor of its intracellular concentration. Even if a drug is applied intracellularly, such as via a micropipette in an electrophysiological assay, it can be difficult to know the local concentration at the target. Thus, the effective concentration of a drug against its target as determined in a cell-free assay, may have limited translation to a cell, tissue, or intact animal. This could lead to under-dosing and result in false negative data. However, higher doses are more likely

to lead to off-target effects that may result in false positive data. Additionally, known off-target effects are often overlooked. Furthermore, high doses of some drugs may lead to negative effects of the drug or its solvent on membrane properties or cell integrity, complicating the interpretation of effects in intact cells, and some compounds may interfere with the assay itself rather than the pathway under study, making the results uninterpretable.

I have attempted to minimize these pitfalls by reviewing the primary literature on the use of each drug, as information provided by a supplier may be biased. Instead of relying on doses reported in other studies, for the most important drugs, I determined dose-response relationships before choosing my experimental drug concentration. To control for solvent effects, vehicle controls were used where appropriate. Furthermore, I substantiated the role of PKA by including two structurally distinct drugs that work by different modes of action.

7.4. Future directions

While I was able to define a 5-HT-dependent mechanism that potentiates OA in nociceptors as well as uncover an unexpected time course of OA in DRG neurons isolated after plantar incision, there are many questions left to explore. Future directions of greatest interest are discussed below.

7.4.1. cAMP signaling in nociceptors – what about POPDC?

POPDC is the most recently discovered cAMP effector. The *Popdc* gene family consists of *Popdc 1*, *Popdc2*, and *Popdc3*. My RNA sequencing data indicates that *Popdc3* and *Popdc2* are expressed in rat DRG, and previous studies showed expression

of *Popdc1* in the DRG and neurons of the spinal cord in embryonic mice (Andrée et al., 2002a, 2002b). Major advances in understanding POPDC function thus far established its important role in regulating heart rate and skeletal muscle regeneration. Importantly, POPDCs are known to interact with ion channels (Schindler and Brand, 2016). For example, in the heart, cAMP modulates the direct interaction of POPDC with the two-pore K^+ channel TREK-1; binding of cAMP to POPDC disrupts its interaction with TREK-1, thereby reducing the plasma membrane localization of TREK-1 and impacting heart rate dynamics (Froese et al., 2012). The potential role of POPDC in neurons remains to be explored and will be an important avenue for future research to further our understanding of cAMP signaling in the peripheral nervous system and whether cAMP-POPDC signaling plays a role in ion channel regulation in sensory neurons.

7.4.2. Role of 5-HT/cAMP signaling in postsurgical pain

A study by Oliveira and colleagues demonstrated that 5-HT levels increased in the paw after plantar incision in mice, and reduction of 5-HT levels in the paw by mast cell depletion was associated with decreased mechanical allodynia post-incision (Oliveira et al., 2011). Oliveira et al. (2011) found that pretreatment with selective 5-HT_3 or 5-HT_{2A} receptor antagonists partially attenuated mechanical allodynia 0.5, 1, and 2 hours after incision, and they were able to achieve complete reversal of mechanical allodynia by coadministration of 5-HT_3 and 5-HT_{2A} antagonists along with a histamine (another mast cell mediator) receptor antagonist. However, this study did not exclude potential roles for 5-HT_4 receptors in other painful effects of incision, notably hyperalgesia and spontaneous pain, which were not examined. Hyperexcitability and OA in nociceptors are implicated in hyperalgesia and spontaneous pain, whereas

mechanical allodynia is thought to be expressed by the activation of A-fiber LTMRs (C. N. Liu et al., 2000; C.-N. Liu et al., 2000; Walters, 2021), which are likely excluded from our electrophysiological studies due to their larger size. Thermal hyperalgesia and spontaneous guarding pain behavior occur after plantar incision, so future studies should examine the contribution of mast cells, 5-HT, and 5-HT_4 receptors to these types of post-incisional pain that are likely driven by hyperexcitability and OA in C-fibers (Djouhri et al., 2006; Wu et al., 2002; Xu and Brennan, 2010). Furthermore, Oliveira and colleagues (2011) showed 5-HT was increased in the paw within one hour post-incision, and levels dropped back down by 48 hours compared to sham-operated animals, but it is not clear how the 5-HT level at 48 hours compares to baseline/naive levels. It will be of interest in future studies to determine whether changes in 5-HT levels only occur locally or if increases can be detected in the blood or CSF as well as how long 5-HT levels remain elevated post-incision compared to baseline.

7.4.3. Why does hyperactivity persist in isolated nociceptors longer than *in vivo* pain and OA?

Studying longer-lasting effects of incision could provide clues to what is involved in the transition from acute to chronic postsurgical pain. One attractive hypothesis that could explain the hyperexcitability observed in isolated nociceptors at 7 and 14 days post-incision when pain behavior is no longer present is that it is a manifestation of hyperalgesic priming. Hyperalgesic priming is a model of pain plasticity in which an initial priming stimulus that sensitizes nociceptors, such as acute tissue damage or inflammation, leads to a primed state after the initial sensitization has apparently

resolved, resulting in an exaggerated nociceptive response to subsequent exposure to an inflammatory test stimulus (Kandasamy and Price, 2015; Price and Inyang, 2015).

Plantar incision has been shown to act as a priming stimulus, resulting in exaggerated allodynia in response to a test intraplantar injection of PGE_2 on postoperative day 14 (Baptista-de-Souza et al., 2020; Burton et al., 2017; Tillu et al., 2012). While a modest dose of an inflammatory mediator such a PGE_2 is typically used experimentally to precipitate primed behavioral responses long after the priming event (e.g. Burton et al., 2017; Khomula et al., 2017; Wang et al., 2018), it is conceivable that the DRG neuron isolation procedure, which involves severing the axons from the cell bodies in the process of DRG removal followed by chemical and physical disruption to dissociate the cells, could also precipitate primed responses, revealing a primed state induced by the incision that is manifested as nociceptor hyperexcitability. This hypothesis can be tested by seeing whether inhibitors of key players known to mediate hyperalgesic priming, such as PKCε (Aley et al., 2000) attenuate signs of hyperexcitability at 7 or 14 days post-incision.

A related hypothesis involves the role of endogenous opioid receptor analgesia that suppresses latent pain sensitization and promotes the return to normal pain thresholds following tissue injury (Campillo et al., 2011; Corder et al., 2013). Inhibition of spinal μ- or κ-opioid receptors (MOR and KOR, respectively) reinstates mechanical allodynia 21 days after plantar incision (Custodio-Patsey et al., 2020). One way to relate the role of endogenous opioids to suppression of nociceptor OA is to test whether MOR or KOR antagonists can reinstate OA in nociceptors *in vivo* at a chronic post-incisional timepoint.

7.4.4. Potential new molecular contributors to postsurgical pain suggested by transcriptional analysis

Of the eight genes I found to be differentially expressed in DRG one week after plantar incision compared to naïve controls, two with functions that plausibly could contribute to nociceptor OA and postsurgical pain are discussed below.

Vip: Vasoactive intestinal peptide (VIP) is a neuropeptide that activates G_s-coupled VPAC receptors and is known to be present in subpopulations of trigeminal ganglion and DRG neurons (Kummer and Heym, 1986; Lazarov, 1994; Xie et al., 2020). Besides neurons, VIP is produced by other cell types, notably immune cells such as mast cells, T cells, and macrophages (Cutz et al., 1978; Delgado and Ganea, 2001; Leceta et al., 1994; Metwali et al., 2002; Wershil et al., 1993). Moreover, macrophages are present in DRG and have been shown to contribute to the initiation and maintenance of pain after spared nerve injury in mice (Yu et al., 2020). However, preliminary data revealed no significant activation of PKA by VIP in primary DRG neurons from naïve rats (A. Garza Carbajal, unpublished observations), suggesting that these cells may exhibit little to no response to this neuropeptide under normal conditions. Rather, the sensory neurons may be producing VIP for release at central terminals, which is in line with early studies showing that VIP is present in Lissauer's tract and Lamina I of the dorsal horn of the spinal cord, where the central axons of nociceptors reach the spinal cord (Purves et al., 2001), in humans, cats, and rats (Anand et al., 1983; Basbaum and Glazer, 1983). Basbaum and Glazer (1983) noted that the staining pattern of VIP was indicative of secretion from Aδ-nociceptors, which represent an important but very small fraction of

DRG neurons. A later study demonstrated increased cAMP levels in the spinal cord linked to release of VIP from primary afferents (Liu and Gintzler, 2003).

In addition to producing VIP, immune cells can also respond to VIP. Accordingly, VIP has also been implicated in neurogenic inflammation, whereby primary afferents release pro-inflammatory neuropeptides into tissues, initiating an inflammatory reaction which can in turn produce painful symptoms. For example, VIP is one of several neuropeptides implicated in neurogenic inflammation that induces psoriasis flares (Al'Abadie et al., 1995; Chan et al., 1997; Kwon et al., 2018; Naukkarinen et al., 1993), which has been linked to VIP's ability to activate immune cells, such as mast cells, to release pro-inflammatory mediators (Choi and Di Nardo, 2018; Lowman et al., 1988; Scholzen et al., 1998). Interestingly, there is evidence that VIP and other neuropeptides can also have anti-inflammatory effects on immune cells. For example, VIP is able to inhibit macrophage production of pro-inflammatory cytokines such as TNF-α and IL-6 (Delgado et al., 2003; Martínez et al., 1998). Such opposing activities may depend upon the physiological context (Baral et al., 2019).

Increased VIP production by DRG neurons after plantar incision should be confirmed, such as by immunostaining or quantitative PCR. To rule out potential effects on DRG neurons of secretion of VIP from other cell types (e.g. macrophages) or possible interaction between DRG neurons via VIP secretion, a simple experiment would be to apply a VPAC receptor antagonist to DRG neurons isolated after incision to see if hyperactivity is attenuated. The contribution of VIP secretion from sensory neurons to post-incisional pain, either at the spinal cord or in peripheral tissue, can be tested by

intrathecal or intraplantar injection, respectively, of a VPAC receptor antagonist prior to behavioral tests.

Stac2: An early study found that genes for Src homology 3 and cysteine rich domain proteins (STAC1 and STAC2) are expressed in a mutually exclusive fashion in mouse DRG neurons, with STAC1 predominantly expressed in peptidergic nociceptors and STAC2 expressed in a subset of nonpeptidergic nociceptors and low threshold mechanoreceptors (Legha et al., 2010). Under the assumption that this expression pattern is similar in rat DRG neurons, we can predict the Stac2 expression detected in my RNA sequencing study of whole DRG tissue occurred in a subset of nociceptors.

STAC2 has been shown to inhibit Ca^{2+}-dependent inactivation of neuronal L-type Ca^{2+} channels, thereby increasing these voltage-dependent Ca^{2+} currents (Polster et al., 2018). While the neuronal function of STAC2 has only been reported in the brain (Polster et al., 2018, 2015), we can predict based on these findings that STAC2 similarly increases L-type Ca^{2+} currents in nociceptors. Unpublished data implicates L-type Ca^{2+} channels in increased ERK signaling in rat DRG neurons after spinal cord injury (A. Garza Carbajal, unpublished observations), which has been linked to persistent nociceptor hyperactivity (Garza Carbajal et al., 2020), and *Stac2* transcripts also appear to be upregulated after spinal cord injury (R. Cuevas-Diaz Duran, A. Garza Carbajal, Y. Li, E. T. Walters, C. W. Dessauer, and J. Wu, unpublished observations). Additionally, a recent study demonstrated L-type Ca^{2+} channel expression and activity in the DRG contributes to the effects of sleep deprivation on delayed recovery from postsurgical mechanical allodynia and heat hyperalgesia in rats (Li et al., 2019). Thus, an exciting

future direction is to investigate whether modulation of L-type Ca^{2+} channel activity by STAC2 contributes to nociceptor OA and spontaneous pain after plantar incision.

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