

IDENTIFICATION OF GPR63 AND GPR153 AS NOVEL MODULATORS OF OPIOID  
ANTINOCICEPTION IN PATHOLOGICAL PAIN AND THEIR ASSOCIATED ROLES IN  
MICROGLIA

by

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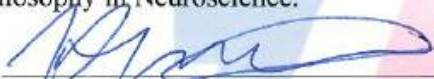
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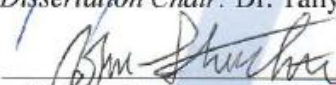
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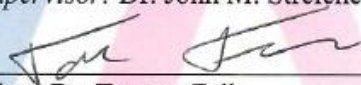
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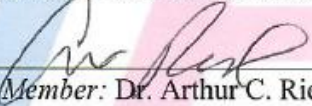
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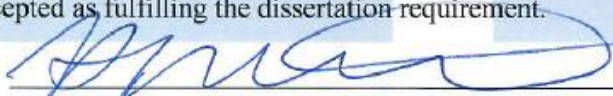
  
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## List of Abbreviations

**2-AG** – 2-arachidonylglycerol  
**5-HT** – 5-hydroxytryptamine; serotonin  
**AAALAC** – Association for Assessment and Accreditation of Laboratory Animal Care  
**ACC** - anterior cingulate cortex  
**AEA** - anandamide  
**AIF1** – allograft inflammatory factor 1  
**ANOVA** – analysis of variance  
**ASIC** – acid-sensing ion channel  
**cAMP** – cyclic adenosine monophosphate  
**CB<sub>1</sub>** – cannabinoid 1 receptor  
**CBD** – cannabidiol  
**CFA** – complete Freund’s adjuvant  
**CGRP** – calcitonin gene-related peptide  
**CIPN** – chemotherapy-induced peripheral neuropathy  
**CMV** – cytomegalovirus  
**CNS** – central nervous system  
**CPSP** – chronic post-surgical pain  
**CUD** – cannabis use disorder  
**DAMGO** – [D-Ala, NMePHe, Gly -ol]-enkephalin  
**DRG** – dorsal root ganglia  
**FADS** – fluorescence-activated droplet sorting  
**GFAP** – glial fibrillary acidic protein  
**GIRK** – G-protein coupled inwardly rectifying K<sup>+</sup> channel  
**GPCR** – G-protein coupled receptor  
**IACUC** – Institutional Animal Care and Use  
**IASP** – International Association for the Study of Pain  
**IBA1** – ionized calcium-binding adapter protein 1  
**IC** – insular cortex  
**ICR** – Institute for Cancer Research  
**IgG** – immunoglobulin G  
**IHC** – immunohistochemistry  
**IL-1 $\beta$**  – interleukin-1 $\beta$   
**IP** – intraperitoneal  
**IT** – intrathecal  
**LC** – locus coeruleus  
**LPS** – lipopolysaccharide  
**MOR** –  $\mu$ -opioid receptor  
**NAGly** – N-arachidonoyl glycine  
**NC** – negative control  
**NIH** – National Institutes of Health  
**NSAID** – non-steroidal anti-inflammatory drug

**OCT** – optimal cutting temperature compound  
**oGPCR** – orphan G-protein coupled receptor  
**OIH** – opioid-induced hyperalgesia  
**p38 MAPK** – p38 mitogen-activated protein kinase  
**PAG** – periaqueductal gray  
**PBS** – phosphate-buffered saline  
**PFA** – paraformaldehyde  
**PFC** – prefrontal cortex  
**PG** – prostaglandin  
**PKA** – protein kinase A  
**PRESTO-Tango** – parallel receptor-ome expression and screening via transcriptional output -  
Tango  
**ROI** – region of interest  
**RVM** – rostroventral medulla  
**S1** – primary somatosensory cortex  
**S1P** – sphingosine-1-phosphate  
**SC** – subcutaneous  
**SNRI** – serotonin-norepinephrine reuptake inhibitor  
**SSRI** – selective serotonin reuptake inhibitor  
**TEV** – tobacco etch virus  
**THC** –  $\Delta^9$ -tetrahydrocannabinol  
**TLR4** – toll-like receptor 4  
**TNF $\alpha$**  – tumor necrosis factor  $\alpha$   
**TRP** – transient receptor potential  
**tTA** – tetracycline transactivator  
**VGCC** – voltage-gated Ca<sup>2+</sup> channel

## Abstract

Pathological pain is a significant public health issue driven by many complex biological mechanisms which are not fully understood, and current pharmacotherapies for long-term pain management are highly variable in their efficacy while also presenting numerous detrimental drawbacks. Many clinical drugs are designed to target GPCRs, but a sizeable proportion of GPCRs remain understudied despite presenting opportunities for new druggable targets. Our lab has chosen four orphan GPCRs – GPR63, GPR141, GPR150, and GPR153 – for investigation in the context of pain and opioid-induced antinociception across multiple mouse models of nociceptive and pathological pain. All four of these targets are known to be expressed in the mouse and human spinal cord according to transcriptomic data from other groups, but their endogenous ligands and functions are poorly understood. We performed CRISPR knockdown of all four receptors in the mouse spinal cord by delivering predesigned CRISPR constructs via the IT route and evaluated their effects in the hot water tail flick assay and CIPN and paw incision pain models with von Frey. We observed no effects of any knockdown on baseline nociceptive responses or the antinociceptive effects of a 3.2 mg/kg screening dose of morphine (SC) in the tail flick assay. GPR63 and GPR153 knockdowns completely ablated the analgesic effects of morphine in the CIPN model, however. Furthermore, GPR153 knockdown yielded a similar result in the paw incision model and showed impairments in recovery from mechanical allodynia post-surgery while GPR63 knockdown had no effect in either regard. Considering that the knockdown of GPR63 and GPR153 had no influence in acute nociceptive pain but did influence behavioral responses to morphine in pathological models, we hypothesized that these receptors may play roles in modulating glial cells

in the spinal cord which are considered to play significant roles in the development and/or maintenance of persistent pathological pain.

Colocalization studies of the expression of GPR63 and GPR153 mRNAs with markers for microglia (*Aif1*) and astrocytes (*Gfap*) demonstrated that both receptors are expressed in approximately 50% of each cell type in the spinal dorsal horns of naïve mice with the exception that GPR153 appears to be expressed in 80-90% of microglia. To interrogate if this expression in glial cells was biologically relevant to the behavioral outcomes we observed previously, we performed selective CRISPR knockdowns targeting GPR63 and GPR153 in microglia or astrocytes alone. We found that when both targets were knocked down in microglia only, the blockade of opioid-induced antinociception and impairments to recovery were recapitulated in CIPN and paw incision models whereas astrocyte-specific knockdown had no effect. From this finding we hypothesized that GPR63 and GPR153 knockdown are enhancing microgliosis in the spinal cord. To test this, we administered minocycline to the spinal cord after GPR63 and GPR153 knockdown to inhibit microglia and were able to rescue opioid analgesia in the CIPN model. Morphological analysis of microglia in GPR63 and GPR153 CRISPR-treated spinal cords also depicted microglia with more amoeboid soma and less complex arborization compared to negative control treatments, both phenotypic indicators of microgliosis. Together, the above findings provide a scientific premise for further investigation of GPR63 and GPR153 as novel targets for analgesic drug discovery through the modulation of microglia in pathological pain states.

## **Chapter 1: Introduction**

## 1.1: Introduction to Pain

Pain sensation is a key sensory system that serves as a protective and learning mechanism for many living organisms, signaling that the body is at risk of serious injury and allowing the organism to react accordingly. As such, acute pain is vital for survival. Chronic pain, however, has become a significant public health issue estimated to affect 24.3% of U.S. adults as of 2023 [1]. Furthermore, the financial burden of chronic pain was estimated at \$560 - \$635 billion in 2010 U.S. dollars [2]. Chronic pain also introduces a severe mental health burden in the United States with 23.9% of adults experiencing chronic pain also reporting incessant anxiety/depressive symptoms [3]. Concerningly, these comorbidities are worsened by the finding that chronic pain sufferers experiencing mental health challenges are less likely to receive effective mental healthcare compared to those without chronic pain [4]. Taking into consideration the breadth of issues that chronic pain imposes on our society, effective pain management strategies are a critical unmet need.

## 1.2: Nociceptive vs. Pathological Pain

To identify means by which we can effectively treat chronic pain, it is vital to define pain and understand its underlying biological mechanisms. According to the International Association for the Study of Pain (IASP), pain is defined as “An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” [5]. Pain that you feel in the event of an injury (e.g. a paper cut or stubbing your toe) can be classified as **nociceptive pain**. This type of pain stems from damage to tissue that activates neurons outside of

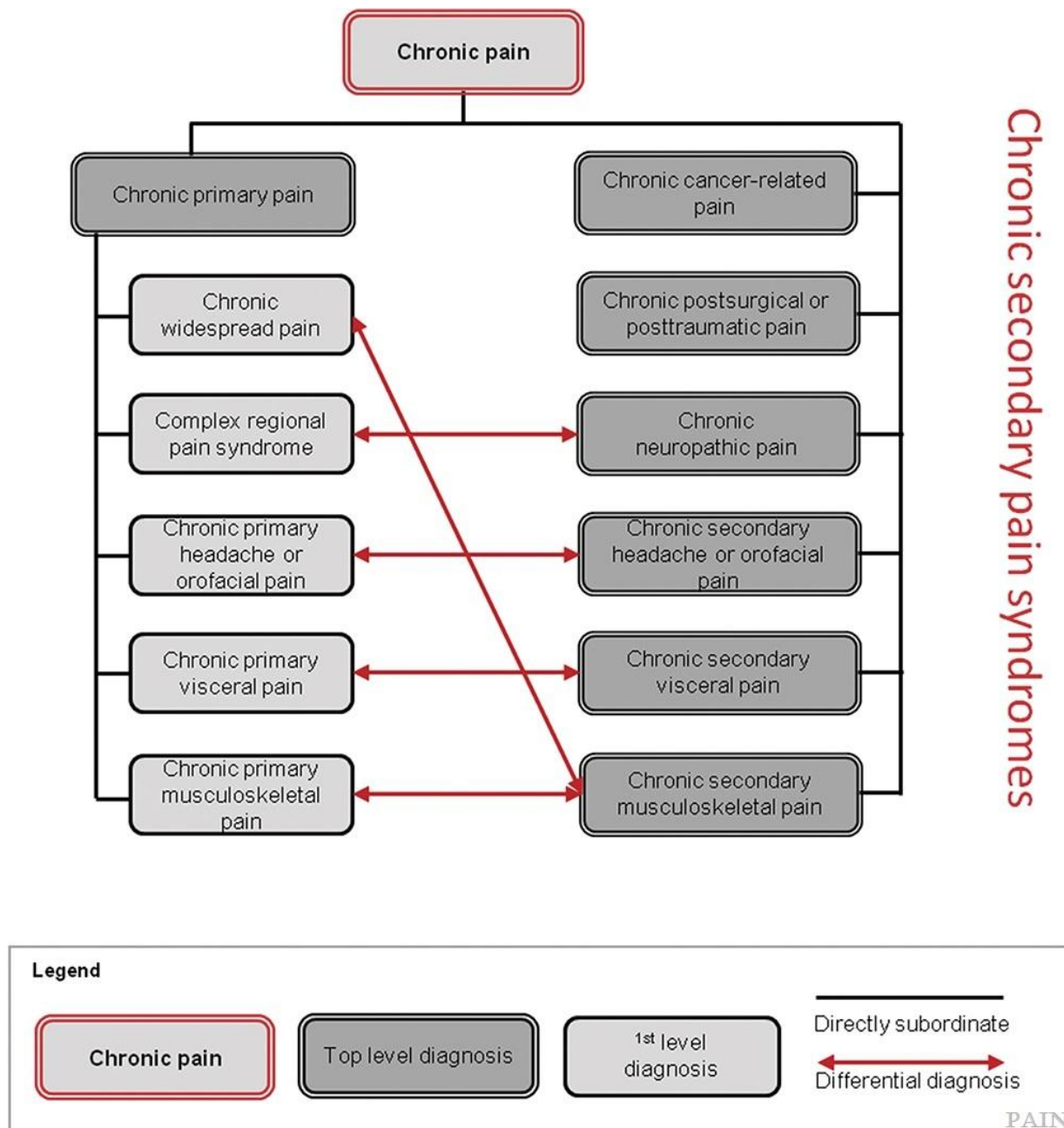
the central nervous system (CNS) responsible for sensing pain known as **nociceptors**. For all intents and purposes, nociceptive pain is a normal physiological response to harmful stimuli and is necessary for survival. The problem that society faces with pain, however, arises when the systems that sense pain become dysfunctional, which results in the chronic pain cases that present themselves in the clinic. Classifying chronic pain introduces challenges when considering effective treatment as its definition according to IASP is somewhat vague, described as “pain that persists or recurs for longer than 3 months.” One type of chronic pain commonly observed in clinical settings is **neuropathic pain** which can result from the dysfunction of the body’s nociceptive pathways due to a wide variety of etiological and pathophysiological causes such as nerve damage, chronic inflammation, and metabolic stressors which can all be introduced by other diseases [6-10] (**Fig. 1.1**). It is important to note, however, that not all cases of chronic pain are neuropathic (e.g., fibromyalgia, low back pain) [11; 12]. Nonetheless, a shared feature of all chronic pain conditions is the presence of maladaptive or dysfunctional activities of the nociceptive pathways or interrelated inflammatory pathways. These perturbation often result in patients presenting with **allodynia**, a condition where normally non-noxious stimuli become painful, and/or **hyperalgesia**, a condition where sensitivity to painful stimuli is augmented [13; 14]. Consequently, treating chronic pain becomes challenging because the health history and context of a patient’s circumstance matters, and there simply cannot be a “one size fits all” solution. With these factors in mind, the scientific community has extensively studied the biological mechanisms that enable the sensation of pain and the many ways it can be dysregulated in an effort to develop a viable repertoire of therapies.

Our lab has performed experiments using various pathological pain models in mice to appropriately assess the viability of novel pain management strategies [15-18]. Mouse models of nociceptive pain generally revolve around noxious thermal and mechanical stimuli being applied to a healthy mouse such as the hot water tail flick, cold acetone, and Randall-Selitto assays and evaluating the antinociceptive effects of experimental treatments. To study pathological pain conditions, however, we must induce an appropriate pain state in animal subjects. For post-operative pain, a common rodent model is the plantar paw incision procedure where the skin and underlying muscle of the mouse's hind paw is incised and sutured to mimic surgery and induce tactile allodynia [19]. Post-surgical pain often becomes persistent with prevalence rates ranging from 5%-85% dependent on the type and location of the surgery [20]. The underlying biological mechanisms by which this occurs can include increased spontaneous firing of nociceptors during recovery through mechanisms of peripheral inflammation like cytokine release and neutrophil infiltration at the surgical site [21-23]. Increased peripheral input then exacerbates nociceptive signaling by central sensitization in which spinal dorsal horn neurons respond more readily to nociceptive inputs (e.g., increase in expression of receptors for excitatory neurotransmitters) or develop their own spontaneous firing [20; 24; 25].

Neuropathic pain models are also commonplace in the development of analgesic drug targets and are designed to mirror what is observed in the clinic. Our most used model for neuropathic pain is the chemotherapy-induced peripheral neuropathy (CIPN) model generated by the scheduled delivery of chemotherapy agents. Most chemotherapy patients report burning, tingling, and numbness in the extremities as a side effect of treatment, and many chemotherapy drugs have been shown to damage peripheral nerves by multiple known and unknown mechanisms

[7; 26-29]. CIPN produces similar outcomes when compared to post-surgical pain at the peripheral and central domains albeit by different mechanisms. Several chemotherapy drugs including paclitaxel, our lab's routine drug for propagating CIPN, stabilize microtubules which often results in peripheral axonal degeneration by disrupting axonal transport [7; 30]. This pathological response often triggers peripheral neuroinflammatory responses, or chemotherapy agents themselves can also directly induce glial reactivity [31; 32]. These outcomes often contribute to central sensitization in similar manner to what was described above in the chronification of post-surgical pain.

Several other models of neuropathic pain are used throughout the literature to model other conditions that produce neuropathy such as sciatic nerve ligation as a nerve injury model or high fat/sugar diets to induce diabetic neuropathy [33; 34]. Inflammatory conditions are also commonly associated with chronic pain and can be modeled in rodents by introducing an immunological challenge with lipopolysaccharide (LPS) or Complete Freund's Adjuvant (CFA), the latter of which producing a reliable model for arthritic pain [10; 35; 36]. To conclude, the wide breadth of chronic and pathological pain cases that present clinically has required the scientific community to creatively adapt to model pain pathologies as accurately as possible for effective pre-clinical evaluations of potential analgesic drugs.



**Figure 1.1: IASP classification of chronic pain.** Due to the complex nature of chronic pain and its underlying etiology and pathophysiology, there is a wide variety of diagnostic considerations for patients. Chronic primary pain is defined as pain restricted to one or more anatomic regions that cannot be effectively classified by another chronic pain condition (e.g., back pain or

fibromyalgia). By contrast, chronic secondary pain classifications are dependent on their relationship to an underlying condition or medical injury as in cases of cancer-related pain, post-surgical pain, or neuropathies to name a few. Diagnoses can be difficult to perform, however, as numerous chronic pain states can present similarities in their symptomologies. This is evidenced by this chart's demonstration of the potential for differential diagnoses between primary and secondary classifications. Figure adapted from [37].

## 1.3: The Neurocircuitry of Pain

### 1.3.1: Nociception at the Periphery

The means by which the nervous system perceives and interacts with pain, hereafter referred to as **nociception**, can be separated into two main phases: pain transduction and pain sensation. Pain transduction begins when peripheral nerve endings receive stimuli that are indicative of tissue stress or damage. These stimuli can manifest themselves in a variety of modalities such as chemical, thermal, or physical insults. Nociceptors that innervate the periphery at the site of injury are equipped with a vast array of receptors that can sense these signals and trigger neuronal firing which conveys this information to the CNS (**Fig. 1.2A**). Common chemical stimuli associated with pain and nociceptor activation are typically related to direct tissue damage and the associated inflammatory milieu such as H<sup>+</sup> ions acting at acid-sensing ion channels (ASICs), bradykinin and prostaglandins acting at their respective G-protein coupled receptors (GPCRs), and neuromodulators such as serotonin (5-HT) [38-41]. Thermal stimuli are sensed by temperature-gated cation channels belonging to the transient receptor potential (TRP) family of ion channels (e.g. TRPV, TRPM, TRPA, etc.) [42; 43]. These channels are vital for sensing hot and cold stimuli and for notifying us of temperatures extreme enough to cause significant damage (i.e. placing your hand on a hot stove or holding dry ice with your bare hands). Interestingly, the TRP receptors can also respond to chemical substances which we perceive as hot or cold (e.g. capsaicin from peppers or menthol from mint) [44; 45]. Mechanical sensation can also often cause pain in the form of strong and deep pressure. This phenomenon is mediated by a group of

mechanically gated ion channels known as the Piezo1/2 receptors that are opened by physical perturbations of the cellular membrane caused by touch and pressure [46].

All these receptors act in a concerted effort to stimulate nociceptors through the influx of the cations  $\text{Na}^+$  and  $\text{Ca}^{2+}$  or by the activity of intracellular secondary messenger systems in the case of the GPCRs. Nociceptors integrate all of these stimuli as they occur in the periphery and fire action potentials along nerve fibers to the spinal cord when sufficient depolarization is achieved. All nociceptor nerve fibers are composed of pseudounipolar neurons whose soma reside just outside of the spinal cord in the dorsal root ganglia (DRG) [47; 48]. These neurons are morphologically unique in that they contain only one axonal projection from the soma which bifurcates and extends to the periphery, where it senses all the aforementioned stimuli, and to the spinal cord thus appearing similar to a bipolar neuron but differing in physiology [47; 48]. Nociceptors are characterized as  $\text{A}\delta$  or C fibers based on their size and degree of myelination as neurons of the former type are larger and myelinated while neurons of the latter type are smaller and unmyelinated [48]. These physical features have important roles in how we sense pain as  $\text{A}\delta$  fibers offer higher conduction velocities compared to C fibers, resulting in our ability to perceive sharp “first pain” and dull, burning “second pain” by  $\text{A}\delta$  and C fibers, respectively [42; 48]. Finally, another key feature in which  $\text{A}\delta$  and C fibers differ is the **pain modalities**, types of pain stimuli, they can transduce.  $\text{A}\delta$  fibers are typically activated by noxious thermal or mechanical stimuli whereas C fibers are equipped with receptors for thermal, mechanical, and chemical stimuli [48-50].

### 1.3.2: Pain Signaling in the Spinal Cord and the Ascending Pain Circuit

Once a nociceptive neuron fires and sends an action potential along its axon to the CNS, that action potential arrives at a synapse in the dorsal horn of the spinal cord. The spinal cord gray matter is organized into laminae I-X where their respective neurons differ in the types of information they are responsible for transmitting [48; 51]. In the context of pain, the primary focus lies in laminae I, II, and V where all nociceptive afferents make their synaptic inputs [48; 51]. These primary afferents interact with dendrites of second order projection neurons in these laminae and release excitatory neurotransmitters such as glutamate, calcitonin gene-related peptide (CGRP), and Substance P [42; 51]. The second order projection neuron in the spinal cord then responds by firing an action potential that conveys pain information up through the brainstem and into the brain.

First, the second order neurons' axons cross the midline of the spinal cord and enter the contralateral white matter where most ascend through the spinothalamic tract. This tract passes through and forms branching synaptic connections with the rostroventral medulla (RVM), pons, and midbrain with until it reaches a third order projection neuron in the thalamus that routes the nociceptive input to other regions of the brain for processing [52; 53] (**Fig. 1.2B**). The spinal cord also has some dedicated local circuits for processing nociceptive inputs and preventing tissue damage known as reflex arcs. The same primary afferent neurons that enter the dorsal horn from the periphery are also known to form synapses on shorter relay neurons, or interneurons, that then excite motor neurons in the ipsilateral ventral gray matter of the spinal cord [54]. These motor neurons are then able to stimulate muscle contraction and move the body part that is at risk.

### 1.3.3: Pain Perception Happens in the Brain

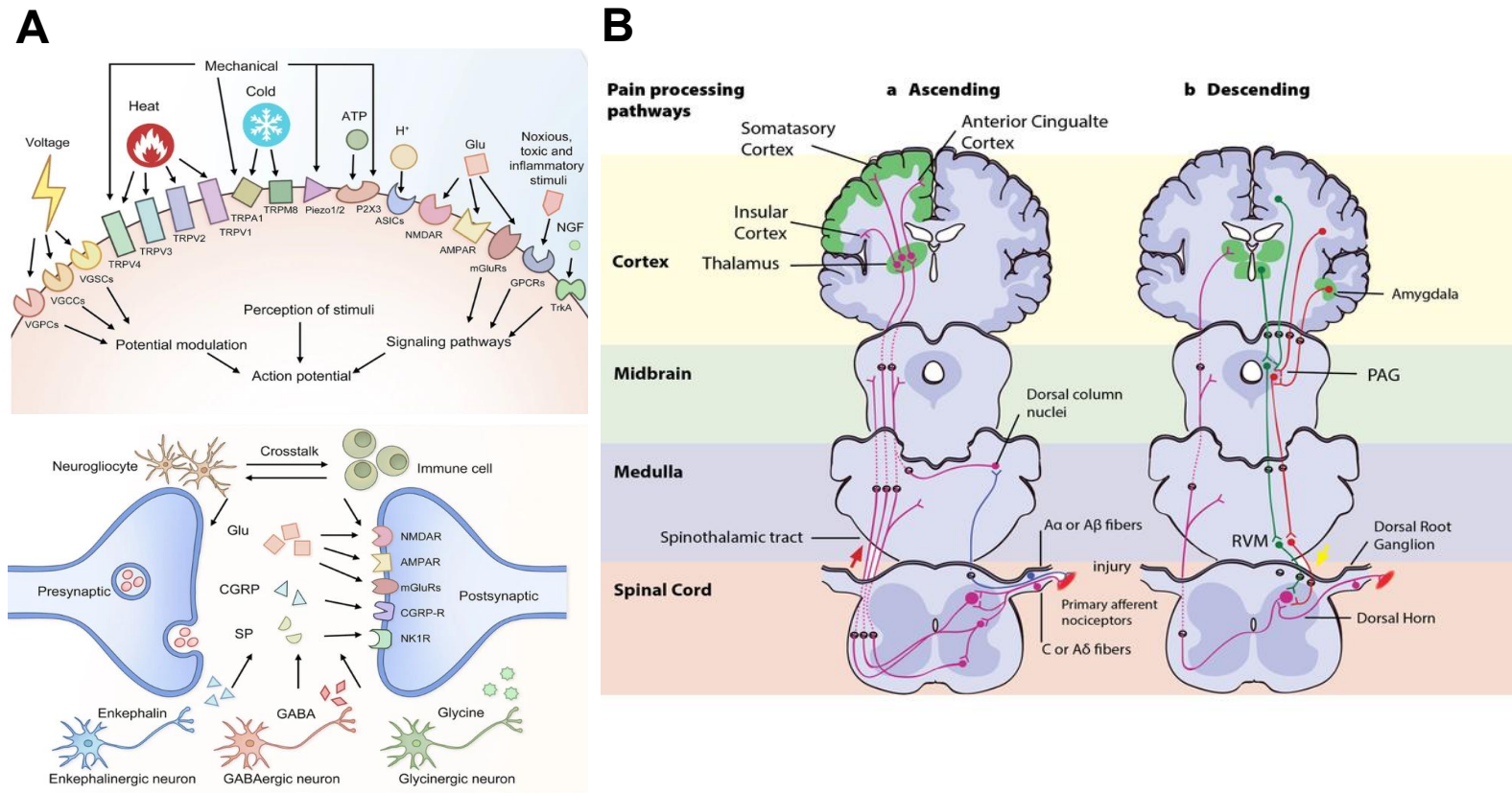
The thalamus is well understood to function as the sensory relay of the brain, receiving most of the incoming sensory inputs transmitted by the spinal cord and cranial nerves. Nociceptive inputs enter the thalamus through its ventroposterior nuclei where they are then redirected to the appropriate cortical regions for processing (**Fig. 1.2B**) [55]. In the cortex, our brains are then able to perform affective processing to the incoming sensory information allowing for higher order processing to make decisions and assign valence to the painful stimulus accordingly.

The main sensory features of pain are evaluated by the primary somatosensory cortex (S1) of the brain which localizes the painful stimulus through its somatotopic mapping and characterizes its intensity [56]. By contrast, affective processing of pain is associated with a more widespread and complex network of cortical structures. The anterior cingulate cortex (ACC) and insular cortex (IC) are well established to have functions correlating to perception of pain unpleasantness and intensity [57; 58]. Pain information is also processed in the amygdala for the assignment of emotion and value to a noxious stimulus [59]. Ultimately, the cortical representation of pain and its associated components allows for decision-making via integration and interaction between these brain regions and the prefrontal cortex (PFC) which is tightly linked to pain processing and activation of the pain modulatory networks of the CNS [60].

### 1.3.4: Neuromodulation of Pain

Inputs from higher brain structures such as the PFC and amygdala are known to send inputs to a region in the brainstem known as the periaqueductal gray (PAG) which in turn is able to modulate pain transmission at the level of the spinal cord by stimulating the rostroventral medulla

(RVM) and the locus coeruleus (LC) [61; 62] (**Fig. 1.2B**). In the PAG-RVM pathway, PAG neurons stimulate GABAergic/opioidergic neurons in the RVM known as “OFF” cells that project to the spinal dorsal horn and inhibit the primary afferent [63; 64]. Conversely, the RVM also has pronociceptive modulatory capabilities through the presence of GABAergic/opioidergic “ON” cells which respond to noxious stimuli and inhibit opioidergic interneurons in the spinal cord needed for pain modulation [64]. The neuromodulatory outcome of both ON and OFF cells is mediated by influencing the release of endogenous opioids such as enkephalin at the synapse that connects the primary afferent and second order projection neurons in the spinal dorsal horn resulting in the inhibition of pronociceptive signaling by acting on  $\mu$ -opioid receptors (MORs) on the pre- and post-synaptic terminals (**Fig. 1.3**). Similarly, the PAG-LC pathway results in the delivery of norepinephrine to the same synapse in the spinal dorsal horn but this neuromodulator only exerts an inhibitory effect on the presynaptic terminal of the synapse via  $\alpha_2$  receptors [61]. The discovery of the endogenous opioid system and its neuromodulatory capacity in the context of pain revolutionized the pharmacological space and provided a pivotal target for pain therapies through the development of opioid drugs.



**Figure 1.2: Mechanisms of pain transduction at the periphery and the central ascending and descending pain pathways. (A)** (Top) Peripheral nociceptors are equipped with a wide variety of receptors that are responsive to noxious stimuli of different modalities such as chemical, heat, mechanical, and electrical signals. Nociceptors differ in that they transduce different modalities of nociceptive inputs depending on their physiology with A $\delta$  fibers being more specifically attuned to thermal or mechanical stimuli whereas C fibers are highly polymodal and typically transduce all modalities. Stimulation of these different receptors from peripheral signals yields overall depolarization of the nociceptor and triggers an action potential. (Bottom) The synaptic terminal of primary nociceptors is located in the dorsal horn of the spinal cord where it interfaces with a second order projection neuron. Excitatory neurotransmitters such as glutamate, CGRP, and

Substance P are released following an action potential to stimulate the second order projection neuron and carry the nociceptive signal further into the CNS. This synapse is also heavily influenced by other factors such as the activity of modulatory interneurons and glial cells. Figure adapted from [65] **(B)** Second order projection neurons receiving nociceptive inputs from the periphery decussate to the contralateral white matter of the spinal cord and ascend through the spinothalamic tract amongst others. These neurons' projections have branching terminations in the brainstem, midbrain, and most importantly the thalamus within the cerebral cortex where it can be conveyed to other cortical regions for sensory and affective processing. Higher-order processing of nociceptive inputs then contributes to executive functions such as decision-making or top-down modulation of pain signals through descending pathways. Figure adapted from [53].

## 1.4: Current Pharmacotherapies for Pain Management and the Opioid Crisis

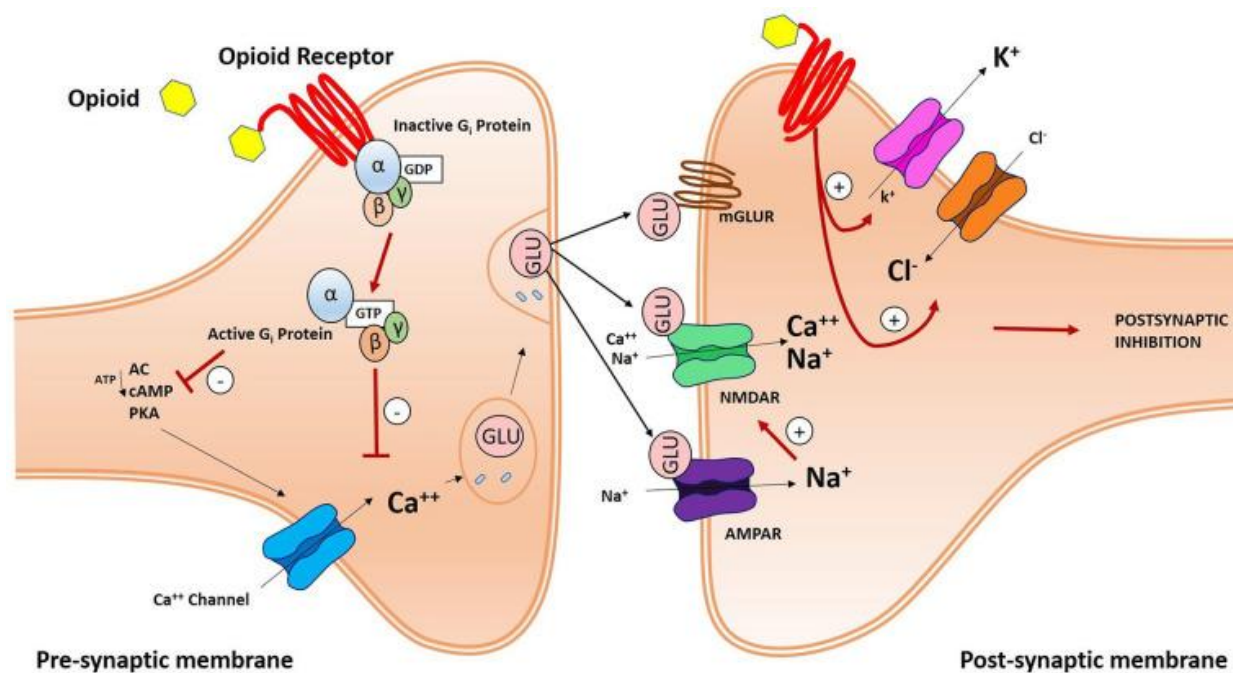
Many of the current pharmacotherapies for pain management consist of several classes of drugs that generally target the ascending pain pathways or the descending modulatory pathways described in **Section 1.3**. These drug classes include opioids, cannabinoids, non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetics, anticonvulsants, and antidepressants. All classes have their own set of advantages and disadvantages when evaluating their clinical effectiveness, and many cases may see combinations of these drugs prescribed to try and maximize pain relief while mitigating the potentially harmful side effects. In this section, we will briefly examine the mechanisms of action of the most common analgesic drugs as well as their benefits and drawbacks.

### 1.4.1: Opioids

As introduced briefly in **Section 1.3.4**, the human body contains endogenous mechanisms to modulate pain with the most significant being the opioid system. Historically, humans have long taken advantage of the opioid system for pain relief with the use of hashish, opium, and similar products derived from *Papaver somniferum* [66]. The pharmaceutical industry has since used morphine, the active analgesic component of opium, to generate numerous semi-synthetic opioid drugs (e.g. heroin, codeine, oxycodone) for various medical applications [66]. Fully synthetic opioid drugs that are structurally unique from morphine (e.g. methadone and fentanyl) have also been developed but still have similar pharmacological effects.

Currently, opioids are considered the “gold standard” for pain relief because they are highly efficacious for some to most pain types. Opioid drugs act on the MOR, the same receptor target of endogenous opioids described above, which is a  $G\alpha_{i/o}$ -coupled GPCR that is expressed throughout the CNS. The MOR is expressed at every node of the ascending and descending pain pathways, from the periphery to the higher brain centers, making the activity of MORs in the pain pathway critical and highly effective for mediating the analgesic properties of opioid drugs [67-69]. MOR stimulation on primary nociceptors inhibits the initial transduction of pain signals through the downstream inhibition of cyclic adenosine monophosphate (cAMP) via adenylyl cyclase. The resulting decrease in cAMP reduces the activity of protein kinase A (PKA) which is necessary for sensitizing cation channels responsible for initiating the nociceptive action potential [68; 70]. At the first synapse in the ascending pain circuit between the primary afferent and second order projection neuron in the spinal cord, MORs are expressed both pre- and post-synaptically which results in a highly effective inhibitory neuromodulator [71] (**Fig. 1.3**). Similar to what is observed in peripheral MOR activation, MOR stimulation at the pre-synaptic terminal and the subsequent decrease in PKA phosphorylation desensitizes voltage-gated  $Ca^{2+}$  channels (VGCCs) which normally respond to an action potential from the primary afferent by allowing the influx of  $Ca^{2+}$  to trigger synaptic vesicle fusion and neurotransmitter release [67; 71]. On the post-synaptic side, the other components of the heterotrimeric G-protein complex,  $G\beta\gamma$ , will stimulate G-protein coupled inwardly rectifying  $K^+$  channels (GIRKs) resulting in  $K^+$  efflux which hyperpolarizes the post-synaptic terminal and blocks the incoming depolarization through the AMPA and NMDA cation channels [67; 71].

Although highly effective at interfering with pain transduction, opioids have been demonstrated to induce several negative side effects including drowsiness, nausea, and constipation [68; 72; 73]. The more concerning issue with opioids, however, stems from their reward/euphoria and subsequent abuse liability, which when combined with their overprescription and misuse, has developed into the opioid crisis present in the United States today [69; 74]. Opioid overdose deaths have risen from approximately 21,000 to over 81,000 per year from 2010 – 2022 with the economic burden of opioid use disorder and loss of life caused by opioid overdose being estimated at 1.02 trillion USD in 2017 [75; 76]. While opioid-induced analgesia is mediated by stimulation of MORs in the ascending pain pathway, opioid dependence is the product of MOR stimulation and the resultant changes of neuronal activity in other brain circuits. The sedative effects produced by opioid consumption are driven by MOR stimulation in the LC which inhibits production of the neurotransmitter norepinephrine responsible for regulating functions such as alertness and respiration [74]. With chronic use of opioids, individuals can develop tolerance triggered by repeated MOR stimulation followed by intracellular mechanisms attempting to reestablish the affected neuron's normal firing tone such as  $\beta$ -arrestin-mediated desensitization and internalization [74; 77]. The transition to opioid addiction is rooted in the opioid system's interactions with the brain's dopaminergic reward pathways resulting in increased dopamine signaling which are also distributed throughout the brain and implicated in modulating various neural processes beyond pain perception and modulation [74; 78; 79].



**Figure 1.3: Mechanism of action of opioid drugs in the spinal dorsal horn.** Opioid receptors are expressed on both pre- and post-synaptic neurons in the spinal cord and thus exert a two-pronged inhibitory effect on nociceptive signaling that solidifies opioid drugs as the gold standard for pain relief. On the pre-synaptic terminal (left), the active  $G\alpha$  subunit inhibits adenylyl cyclase which reduces cAMP production needed for PKA phosphorylation. The absence of PKA phosphorylation results in the desensitization of VGCCs and loss of  $\text{Ca}^{2+}$  influx in response to action potentials generated by nociceptive signals originating in the periphery. As a result, synaptic vesicles containing excitatory neurotransmitters (e.g., glutamate, CGRP, Substance P) cannot fuse to the pre-synaptic membrane and release their contents into the synaptic cleft. On the post-synaptic terminal (right), a similar outcome is observed as reductions in PKA activity desensitize post-synaptic cation channels (e.g., AMPARs and NMDARs). Additionally, the  $G\beta\gamma$  subunit

triggers  $K^+$  efflux through GIRKs to yield hyperpolarization of the post-synaptic neuron and shunt any incoming depolarizing currents. Figure adapted from [71].

#### 1.4.2: Cannabinoids

The endocannabinoid system is another endogenous neuromodulatory system that is highly implicated in regulating pain sensation. The cannabinoid 1 (CB<sub>1</sub>) receptor, like MOR, is a G $\alpha_{i/o}$ -coupled GPCR heavily expressed throughout the CNS and peripheral nerves, and as such, inhibits neural pathways responsible for pain transduction and perception when stimulated by the endogenous ligands 2-arachidonylglycerol (2-AG) and anandamide (AEA) [80]. Similar to the discovery of opium from the poppy plant, *Cannabis sativa* has a longstanding history of human use for a variety of purposes but is most recognized for its medicinal and recreational applications [81; 82]. The two main phytocannabinoids found in *C. sativa*,  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD), act as an agonist and negative allosteric modulator of the CB<sub>1</sub> receptor, respectively, with the former being the main driver of the cannabinoid tetrad effects (i.e., analgesia, catalepsy, hypothermia, and hypolocomotion) mediated by CB<sub>1</sub> activation [83; 84]. The use of medicinal cannabis has become more prominent worldwide due to its analgesic properties and evidence supporting benefits in other disease states, but not without legal and ethical challenges [85]. Although highly investigated and self-reported as having therapeutic capacity, a significant drawback of medicinal cannabis lies in the psychoactive effects produced by THC and the widespread expression of CB<sub>1</sub> receptors throughout the cerebral cortex [86]. Furthermore, cannabis is still classified as a Schedule I substance by the DEA and is considered an illicit substance according to US federal law which has hindered research efforts, but it has been legalized for medicinal and/or recreational use in the majority of US states [85; 87]. Public opinion regarding cannabis use has shifted in favor of its legalization over the last decade, but arguments to reschedule cannabis, THC, and CBD have been challenged by the increasing prevalence of

cannabis use disorder (CUD) and links to the dopaminergic reward system supporting significant abuse liability [88-90]. To fully elucidate and maximize the therapeutic potential of cannabis and the endocannabinoid system while minimizing the deleterious side effects, THC and CBD continue to be heavily studied as well as other compounds found in *C. sativa* (e.g., minor cannabinoids and terpenes) which have shown some efficacy in producing antinociception in chronic pain models through CB- and non-CB-mediated pharmacological mechanisms [17; 91-95].

#### 1.4.3: Serotonergic Drugs and Anticonvulsants

Also described briefly in **Section 1.3.4**, serotonin is another neurotransmitter whose signaling modulates nociceptive inputs via the descending pathways. The serotonergic system within the body is highly promiscuous, consisting of 15 total receptors across seven families (5-HT<sub>1-7</sub>; all GPCRs except the cation channel 5-HT<sub>3</sub>) and respective subtypes within those families [96; 97]. Expression of 5-HT receptors is also broad and linked to many biological functions ranging from mood regulation to gastrointestinal motility to sexual function [96]. Currently, many drugs that have originally been indicated for treating depression have since been repurposed and prescribed in the clinic for pain management. This class of drugs includes tricyclics (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and selective serotonin reuptake inhibitors (SSRIs), which are all commonly used as antidepressants [98; 99]. By virtue of their common application, they also share a common mechanism of action by blocking the reuptake of 5-HT via serotonin transporters of the presynaptic terminal and local glia and thus increasing 5-HT signaling by prolonging its time spent in the synaptic cleft [100]. This activity has been shown to have antinociceptive effects in clinical cases and are seeing more common use in cases of craniofacial

pain [101; 102]. A key limitation to note when targeting 5-HT signaling, however, lies in the aforementioned promiscuity of the serotonergic system which can result in widespread undesirable side effects.

Anticonvulsant drugs have also seen repurposing in attempts to treat cases of persistent pain, despite their primary use as anti-epileptics [103-106]. These most commonly include the gabapentinoids and benzodiazepines amongst others, which act primarily by depressing the CNS, decreasing neuronal firing broadly to reduce pronociceptive signaling but also increasing inhibition in other neural circuits. Gabapentinoids such as gabapentin and pregabalin have a unique mechanism of action in that they specifically bind the  $\alpha 2\delta$  subunit of VGCCs in the presynaptic terminal of neurons [105; 107-109]. This subunit is critical for the direction and insertion of VGCCs into the plasma membrane, but when inhibited by gabapentinoids results in the ablation of  $\text{Ca}^{2+}$  influx required for synaptic vesicle fusion and neurotransmitter release when an action potential is fired [107; 110]. Benzodiazepines differ in that they bind to allosteric sites of the  $\text{GABA}_A$  receptor, an anion channel, and potentiate the effect of the main inhibitory transmitter of the CNS, GABA [103; 106].

#### 1.4.4: NSAIDs and Local Anesthetics

Thus far, the drugs that have been discussed in the context of pain management must be acquired by a medical professional's prescription and may require strictly controlled dosing, but certain drugs with little to no abuse liability and minimal side effects have been made available to the public in over-the-counter (OTC) pharmacies. NSAIDs – the most common being ibuprofen, acetaminophen, and aspirin – act specifically by inhibiting the production of prostaglandins (PGs)

and other proteins of the COX-1/2 biosynthetic pathways, which stimulate neurons of our nociceptive pathways in both the CNS and the periphery [111; 112]. Generally, PGs such as PGE<sub>2</sub> are considered pronociceptive as their associated receptors (EP<sub>1-4</sub>) primarily yield hyperexcitability of nociceptive neurons apart from the G $\alpha_{i/o}$ -coupled EP<sub>3</sub> receptor [39]. Ibuprofen and aspirin are both non-selective inhibitors of both COX-1/2 enzymes which prevents the conversion of arachidonic acid to the biosynthetic precursors of PGs [113; 114]. Acetaminophen is believed to function similarly as a selective COX-2 inhibitor but is also considered to have several other potential mechanisms of action by which it produces antinociception; considering the lack of a clear mechanism of action for acetaminophen, it is interesting to note that it would not pass through present day FDA requirements for a new drug despite its longstanding use in the general population and lack of significant adverse effects when used appropriately [115; 116]. Benefits of NSAIDs come in the form of their ease of access, ease of use through oral dosing, and generally favorable pharmacokinetics but also come with drawbacks that arise with chronic use (e.g. gastric ulcers, stroke, interactions with other drugs, etc.) that make them unsuitable for treating chronic pain [117; 118]. COX-1 is generally viewed as being involved in the more protective applications of PG synthesis like gastrointestinal mucus production whereas COX-2 is implicated more in inflammatory processes, but the non-selective nature of some NSAIDs combined with extended use can lead to harmful side effects [111; 112; 119; 120].

Other readily available options for pain management include local anesthetic drugs such as lidocaine and benzocaine with topical delivery methods (i.e., patches, creams, sprays, etc.) while also used in higher doses for clinical procedures and minor surgeries. Pharmacologically, local anesthetics block Na<sup>+</sup> channels on neurons and prevent the propagation of action potentials [121].

This interaction is reversible and short-lived, however, and their topical application limits their use to superficial areas making their use in chronic pain management unfeasible.

#### 1.4.5: Limitations of the Current Pharmacotherapies for Pain

All of the above drug classes have demonstrated effectiveness in producing analgesia in patients; however, they all present significant drawbacks such that the need for identifying novel drug targets for pain management remains. As described above, long-term opioid use presents a significant risk of tolerance which impairs their analgesic capacity. Furthermore, chronic opioid use in patients with polyneuropathies has been correlated with reports of pain beyond the prescription course suggesting their ineffectiveness long-term along with comorbidities such as depression and chemical dependence [122; 123]. Cannabinoids show significant efficacy in pain relief, but the nature of the endocannabinoid system and common modes of cannabis consumption present deleterious side effects as well. THC and CBD are known to have highly undesirable psychoactive side effects that can impair daily functioning and complicate long-term applications of medicinal cannabis as they introduces risks in the form of impairments to motor and executive function [86; 124]. Chronic cannabis use has also been linked to the development of respiratory or vascular conditions (e.g., bronchitis, myocardial infarction, stroke, etc.) [125; 126]. Drugs targeting the serotonergic system such as SSRIs and anticonvulsants like gabapentinoids have proven analgesic capacities in the clinic, but clinical meta-analyses suggest that they may not be viable for most patients as demonstrated by high numbers needed to treat (NNT) [100; 105]. On the contrary, TCAs are highly efficacious with low NNTs, but targeting 5-HT neurotransmission can lead to undesirable side effects (e.g., dizziness, constipation) and increased overdose risk [100;

127]. Lastly, analgesia from OTC NSAIDs is often viable in acute settings, but their nature of targeting inflammatory pathways specifically makes them ineffective at treating conditions lacking a significant inflammatory component while also increasing risks in adverse events in patients with pre-existing gastrointestinal or cardiovascular issues [128].

## 1.5: Orphan GPCRs and Their Potential as Drug Targets

The human genome contains over 800 GPCRs [129]. Of the non-olfactory GPCRs, 100 – 140 have been studied and confirmed as drug targets, including the opioid, cannabinoid, and serotonin receptors described thus far [130; 131]. There remains significant unexplored space within the human GPCRome, however, as it is estimated that approximately 30% of non-olfactory GPCRs are still classified as orphan receptors – receptors with no known ligands and/or biological functions [130-132]. Consequently, orphan GPCRs (oGPCRs) are highly attractive for identifying new druggable targets to a young and ambitious – though some might say foolish – scientist but also for the novelty of uncovering previously unknown insights into human biology.

Many oGPCRs have been studied or are actively being studied for potential treatment or implication in a variety of neurological disease states. For instance, the oGPCRs GPR3, GPR50, GPR55, and GPR84 have all been linked as potential targets for Alzheimer's disease, a notoriously difficult disease state to study with no known cure, based on factors such as changes in expression levels and patterns in the disease state, relationships to other receptor targets, and/or reported changes in pathology in response to oGPCR activity [132-136]. Some oGPCRs have been considered key regulators of neurodevelopment such as GPR37 and GPR85 along with

associations to Parkinson's disease and schizophrenia [137; 138]. GPR55 has also been tied to drug-seeking behavior, Parkinson's, neuroinflammation, and the endocannabinoid system [133; 139-142]. An issue remains, however, in that once oGPCRs are implicated in one disease state, they are often pursued across other disease states as evidenced by the repetition of some oGPCRs listed above across several areas of interest. While this approach is valid considering well-studied GPCRs are involved in regulating various functions of the CNS (i.e., the opioid, endocannabinoid, and serotonergic systems), a lot of ground still remains to be uncovered in studying oGPCRs. Broader transcriptomic and phylogenetic analyses are also commonplace in the oGPCR space as researchers try to generate hypotheses on potential functions based on expression mapping in the brain or structural relationships with other receptor families [143-146].

While showing promise in multiple disciplines within neuroscience, oGPCRs have been investigated in the context of interactions with the opioid system and/or chronic pain states. GPR171 has been reported to modulate nociceptors in mouse models of pathological pain which has been corroborated by other groups, albeit with the caveat of potential sex differences [147-149]. This same receptor is also described as antinociceptive via interplay with the opioid system without augmenting the harmful side effects of opioid drugs like morphine [150]. By contrast, some evidence of downstream signaling of GPR88 counteracting opioid receptors amongst others in the striatum has also been published [151]. GPR160 is speculated to be pronociceptive with its knockout resulting in failure to develop mechanical allodynia in a mouse model of neuropathic pain [152]. In another case, GPR83 knockdown mitigated the activity of cultured neurons stimulated with capsaicin and dampened pathological pain phenotypes in inflammatory and neuropathic pain models [153]. Pharmacological targeting of GPR40 by lipid mediators suspected

to be analgesic demonstrated effectiveness in generating antinociception in response to a chemical insult in mice [154]. In cases of diabetic neuropathy, GPR177 is described as a key contributor through its signaling mechanisms increasing activation of key nociceptive signal transducers like TRPV1 [155]. Lastly, reported GPR132 mediation of macrophage infiltration of the nervous system in response to injury may provide insights into the intersection between the peripheral immune system and the propagation of neuropathic pain states [156]. Considering the above findings, it is evident that oGPCRs offer several candidates for targeting numerous diseases and conditions of the nervous system including chronic pain. Beyond their therapeutic potential, they also present abundant opportunity for basic discovery and developing a better understanding of the machinations of the CNS.

## 1.6: Significance and Goals

In summary, chronic pain is a significant medical stressor within the US and on a worldwide scale. The nociceptive pathways responsible for pain sensation and perception along with the available pharmacological targets for pain management have been extensively studied, but many of the options available to patients today lack long-term efficacy, reduce quality of life in other aspects, or a combination of both. As such, it is critical to continue research efforts to identify new drug targets and gain new insights into the underpinnings of pathological pain states to develop more appropriate pharmacotherapies. Orphan GPCRs remain an untapped resource of potential pharmacological targets that are attractively situated for novel drug discovery and biomedical research efforts.

**Overall goals of this work:** This work presents a behavioral screen of four oGPCRs – GPR63, GPR141, GPR150, and GPR153 – to identify if any are suitable candidates for drug discovery in nociceptive and pathological pain states using mouse models and the application of opioid drugs. After identifying promising candidates for further investigation, efforts to determine how these receptors influence pathological pain and their role in the current understanding of the neurobiology of pain will be made. Ultimately, the data presented here is intended to provide a framework for long-term study of highly novel and understudied oGPCRs and their evaluation as potential drug targets for pharmacological pain management.

**Chapter 2: A Behavioral Screen of Four Orphan GPCRs in  
the Mouse Spinal Cord in the Context of Pain and Opioid-  
Induced Antinociception**

## 2.1: Introduction

Effective treatments for chronic pain are a critical, unmet need as chronic pain is estimated to affect 1 in 5 adults worldwide [157]. The International Association for the Study of Pain has defined chronic pain broadly as pain that persists for at least three months; thus, the etiologies that contribute to chronic pain can vary widely [158]. The biological mechanisms underlying chronic pain are not fully understood, complicating the issue of developing therapies for pain management. Pharmacotherapies designed for the management of chronic pain are currently dominated by opioid drugs thanks to their high efficacy in providing pain relief. While they are valuable analgesics in scenarios of acute pain, their value in the context of chronic pain is highly contested due to their abuse liability and side effects like tolerance, leading to loss of long-term effectiveness and eventual overdose and death in some patients<sup>[159]</sup>. These drawbacks are exacerbated by other side effects that decrease patient quality of life (e.g., drowsiness, constipation, nausea). Thus, pain research has shifted in the direction of alternative pharmacological targets for the treatment of chronic pain to avoid the adverse effects of opioids.

A significant proportion (approximately 35%) of clinical drugs, including opioids, are designed to target GPCRs, and GPCRs continue to be extensively studied for drug discovery purposes today [130; 160; 161]. Presently there are many GPCRs classified as orphan receptors – receptors of which the endogenous ligands are unknown and specific functions are unclear [131]. Several oGPCRs are known to be expressed throughout the CNS [143; 162]. Furthermore, some have been implicated in the transmission or modulation of pain [147; 162]. Consequently, the lack of knowledge regarding these GPCRs and their potential as novel targets for analgesic drugs makes them attractive for pharmacological investigation. Additionally, there has been recent success in

targeting oGPCRs with novel ligands to enhance morphine-induced analgesia and potentially reduce opioid dosage or replace opioids altogether, further exemplifying their potential as analgesic drug targets [148; 150].

The goal of this work is to identify new oGPCRs that modulate pain or the endogenous opioid system and determine their roles in the underlying pain neurocircuitry. In an effort to maximize the novelty of our effort, four oGPCRs with little to no publication record and relevant expression in the CNS were chosen for investigation – GPR63, GPR141, GPR150, and GPR153. While expression in the CNS has been confirmed for all targets by RNASeq using human tissue and is comparable to  $\mu$ -opioid receptor expression as a reference point, their endogenous ligands and functions are essentially unknown [163]. Previous investigation of GPR63 reported sphingosine-1-phosphate (S1P) and dioleoylphosphatidic acid as potential low affinity agonists [164]. S1P signaling is currently being investigated as a non-opioid target for pain therapeutics because of its role in neuroinflammation that contributes to the precipitation of chronic pain [165]. The role of S1P in neuroinflammation is heavily mediated by astrocytes and microglia where its receptors are highly expressed, and previous expression mapping of oGPCRs in the mouse brain indicates that GPR63 may be expressed in astrocytes [143; 165]. These findings suggest that GPR63 holds a similar role in cellular signaling pertaining to neuroinflammation and/or central sensitization that contributes to chronic pain. Other reports suggest that GPR63 is important for neurodevelopment as well, but this dataset is limited [166; 167].

GPR141 is another receptor potentially implicated in neurodevelopment as it is dysregulated in cells with mutated Ras/MAPK signaling – a pathway responsible for regulating cell growth and maturation [168]. Phylogenetic analysis examining structural and sequence

homology of GPR141 suggests N-arachidonoyl glycine (NAGly) as an endogenous ligand based on the receptor's similarity to GPR18, of which NAGly is a verified ligand [144; 169]. Previous work implicates NAGly as having analgesic properties in a neuropathic pain model when applied to the spinal cord via intrathecal injection [170]. Considering these findings, GPR141 may also be involved in the neurocircuitry that underlies pain.

GPR150 is a very poorly characterized GPCR with potential sequence and structural homology to receptors sensitive to gonadotropin-releasing hormone [144]. This may suggest that GPR150 is involved in regulating the HPA axis, a biological system which potentially plays a role in the transition from acute to chronic pain states [171]. Contradictory RNASeq data regarding the presence of GPR150 expression in the nervous system makes this receptor's role difficult to hypothesize, however [143; 163].

Lastly, GPR153 has been characterized to be phylogenetically similar to the  $\beta$ 2 adrenergic receptor and various serotonin receptors [144]. It has also been reported to be expressed throughout the cortex and thalamus as well as microglia in the mouse brain [143; 172]. Other groups have linked GPR153 to the regulation of luteinizing hormone and follicle stimulating hormone release in the bovine anterior pituitary, showed co-expression with the gonadotropin releasing hormone receptor in the cell membrane, and reported changes in expression levels throughout different phases of the estrous cycle [173-175]. None of these findings provide any concrete mechanistic insight into the regulatory functions of GPR153. Changes in GPR153 expression in the kidney were also reported in a rat model of diabetic nephropathy [176].

The current body of literature provides evidence that all targets of interest are expressed in the CNS and have some links to regulatory function of the nervous system. No information is

presented, however, providing a meaningful link between any target oGPCR and the regulation of pain or the endogenous opioid system. In this chapter, we have performed a set of behavioral screening experiments with the intent of linking any of our target receptors to the modulation of opioid-induced antinociception and/or pathological pain.

## 2.2: Materials and Methods

### **Drugs**

Morphine sulfate pentahydrate was obtained through the National Institute on Drug Abuse Drug Supply Program and distributed through the Research Triangle Institute. Paclitaxel (Cat. No. AAJ6273403, Lot Q25K025) was purchased from Fisher Scientific (Waltham, MA). Gentamicin sulfate (Cat. No. 1098195) was purchased from Henry Schein (Melville, NY). Morphine and paclitaxel were crystalline white solids and stored by manufacturer's recommendations (morphine at 20-25°C; paclitaxel at -20°C). Gentamicin was received in 2 mL vials at a concentration of 40 mg/mL in USP Saline. Morphine was prepared fresh for every experiment in USP Saline. Paclitaxel was prepared as a stock solution in a 1:1 mixture of Kolliphor EL (Fisher Scientific, Cat. No. 50-165-7076) and absolute ethanol (Fisher Scientific, Cat. No. BP28184) and stored at -20°C. Gentamicin was prepared as a stock solution in USP Saline and stored at -20°C.

### **Animals**

Male and female CD-1 mice aged 5-8 weeks from Charles River Laboratories (Wilmington, MA) were used for all experiments. CD-1 [(also known as Institute for Cancer Research (ICR)] mice

are commonly used in opioid research and in our previous work as an outbred mouse strain with strong responses to opioid drugs [15-18]. Male and female mice were used in approximately equal numbers for all behavioral experiments. No sex differences were observed in behavioral experiments; thus, all data shown for behavioral experiments was pooled from male and female cohorts. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) – accredited vivarium at the University of Arizona where they were acclimated for at least five days after shipment before being used for experiments. Twelve-hour light and dark cycles were maintained with food (standard chow) and water available *ad libitum*. Mice were housed with no more than five per cage. Animal monitoring was provided daily, including after surgical procedures, by trained veterinary staff. All experiments were performed in accordance with Institutional Animal Care and Use (IACUC) – approved protocols at the University of Arizona and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### **CRISPR and siRNA Knockdown**

All-in-one predesigned CRISPR constructs containing sgRNA and Cas9 expression cassettes were obtained from Genecopoeia (Rockville, MD) (**Fig. 2.1**). Knockdown constructs were designed to target mouse *Gpr63* (Cat. No. MCP236602-CG12-3-B), mouse *Gpr141* (Cat. No. MCP300299-CG12-3-B), mouse *Gpr150* (Cat. No. MCP241327-CG12-3-B), or mouse *Gpr153* (#MCP242236-CG12-3-B) genes. As a negative control (NC), a universal vector containing all the same elements as the above constructs and a nontargeting sgRNA was used. All constructs arrived in the form of transformed bacterial stabs. Bacterial stocks were prepared by

streaking the initial stabs on LB agar/ampicillin (50 µg/mL, 37°C) plates, culturing single colonies in LB broth with ampicillin (37°C, shaking), and preparing frozen stocks in 1:1 broth:glycerol solution, stored at -80°C. Bacteria stocks were recovered from frozen stocks by scraping the top of the frozen stock and incubating in 200 µL of S.O.C. Medium (Fisher Scientific, Cat. No. 15-544-034) for at least 5 hours but no more than 24 hours in an incubated shaker at 37°C. S.O.C. Medium containing propagated bacteria was plated on sterilized LB Agar (Fisher Scientific, Cat. No. BP9724-500) containing 50 µg/mL ampicillin (Fisher Scientific, Cat. No. BP1760-25) and incubated overnight at 37°C. Single colonies of plated bacteria were isolated and added to individual flasks containing 200 mL of sterilized LB Broth (Fisher Scientific, Cat. No. BP1426-500) with ampicillin and incubated overnight in an incubated shaker at 37°C. DNA plasmids containing CRISPR constructs were then isolated using EndoFree® Plasmid Maxi Kits (Qiagen, Cat. No. 12362) following the manufacturer's protocol.

All constructs were administered at a dose of 1 µg DNA in a 5 µL volume (0.2 µg/µL) with GenJet *In Vivo* Transfection Reagent (SignaGen Laboratories, Cat. No. SL100500) according to the manufacturer's protocol. In brief, 20 µg of DNA (volume dependent on concentration of CRISPR construct DNA stock) was combined with 30 µL of 10% glucose in water and diluted to a total volume of 50µL with nuclease-free water (Fisher Scientific, Cat. No. PR-P1193). Separately, 30 µL of GenJet *In Vivo* Transfection Reagent (1:1.5 ratio of DNA in µg to transfection reagent in µL) was combined with 20 µL of 10% glucose in water. The final concentration of the combined solution must be 5% glucose. Both solutions were combined, vortexed, and incubated for 10 minutes at room temperature then immediately administered to CD-1 mice. Constructs were delivered via the intrathecal (IT) route twice daily with at least 2 hours between injections for three

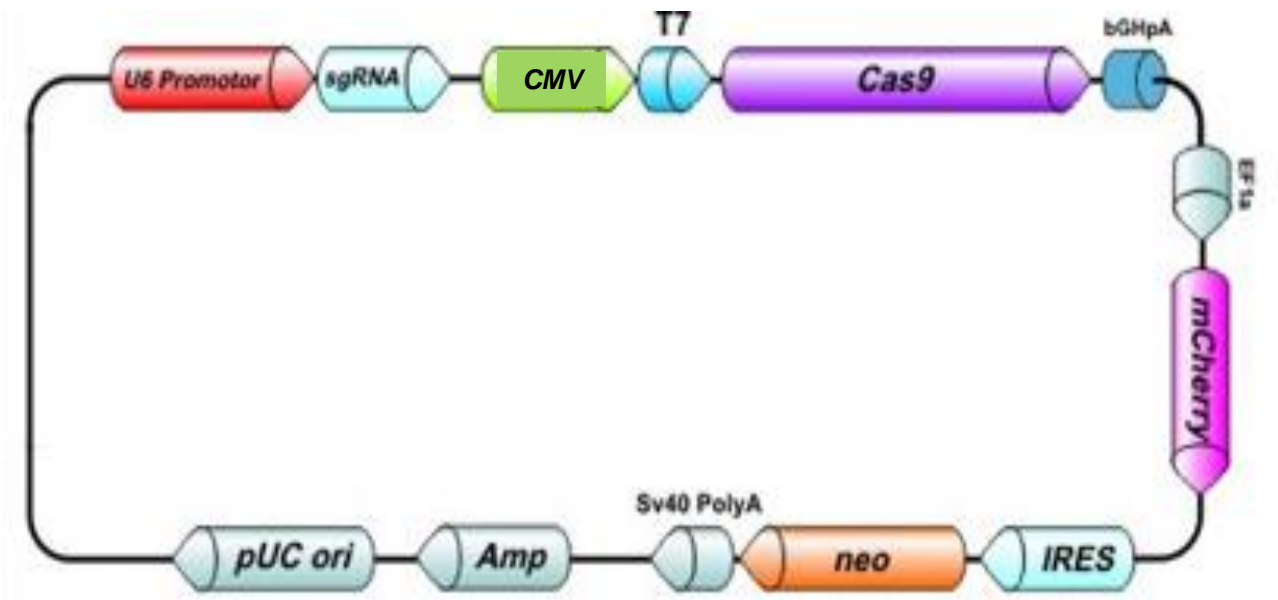
consecutive days resulting in a total 6 µg dose. All behavioral experiments or tissue collections were conducted on day 10 following the first IT injection.

For siRNA knockdowns, targeted siRNA duplex oligonucleotides were designed by Integrated DNA Technologies (IDT; Newark, NJ). Custom duplex oligonucleotides were designed to target mouse *Gpr63* and *Gpr153*:

Mouse *Gpr63*, Reference #444983252: (5'-AUU AUU AGU AUC GAU AGG UUU CUG A-3',  
5'-UCA GAA ACC UAU CGA UAC UAA UAA UGA-3')

Mouse *Gpr153*, Reference #444983255: (5'-GUA UUG GAG CGC UCU CUU GAC UAT A-3',  
5'-UAU AGU CAA GAG AGC GCU CCA AUA CCA-3')

As a negative control, a duplex oligonucleotide that does not recognize the sequence of any gene in the human, rat, or mouse transcriptomes was used (IDT, Cat. No. 51-01-14-03). Oligonucleotides arrived as lyophilized solids and were dissolved in nuclease-free water to a concentration of 100 µM. The transfection complex for siRNA was mixed in identical fashion to that of the CRISPR constructs, ensuring that siRNA and GenJet *In Vivo* Transfection Reagent are mixed in a 1:1.5 ratio alongside a 5% glucose final concentration. The siRNA transfection mixture was incubated for 10 minutes at room temperature and administered immediately via the IT route. Injections took place twice daily with at least 2 hours between injections for two consecutive days to yield a total siRNA dose of 4 µg. All behavioral experiments or tissue collections were conducted on Day 4 following the first IT injection.



**Figure 2.1: Schematic of CRISPR-Cas9 construct used for *in vivo* CRISPR knockdown.** The CRISPR construct used in this study for *in vivo* CRISPR knockdown of oGPCR targets has two main components necessary for gene-targeted knockdown. Each construct contained an sgRNA targeted to the receptor of interest with its expression driven by a universal U6 promoter. Additionally, the expression of the Cas9 protein was driven by a cytomegalovirus (CMV) promoter to be expressed in all cell types.

## **Behavioral Experiments**

Prior to behavioral experiments, animals were acclimated to the experimental setting (i.e., testing room and/or apparatus) for 30-60 minutes before any procedure. Mice were randomly block-assigned to treatment group by cage. Behavioral testing took place at the approximate same time of day between experiments while minimizing environmental factors (e.g., noise, personnel, and scents). Testing apparatuses were cleaned after each use. The experimenter was blinded to treatment groups for all behavioral experiments through coded animal cage labels generated by another laboratory member; decoding/unblinding occurred only after data collection was complete.

## **Tail Flick Assay**

Tail flick baselines were determined in a 52°C warm water tail flick assay with a 10 sec cutoff time as in our previous work [15-18]. On Day 10 of the CRISPR treatment timeline described above, post-CRISPR tail flick responses were measured followed by subcutaneous (SC) injection of 3.2 mg/kg morphine. Following morphine administration, tail flick latencies were measured over a two-hour time course. No animals were excluded from these studies.

For siRNA experiments, on Day 4 the post-siRNA tail flick responses were measured followed by SC injection of 3.2 mg/kg morphine. Following morphine administration, tail flick latencies were measured over a two-hour time course. No animals were excluded from these studies.

### **Chemotherapy-Induced Peripheral Neuropathy and Mechanical Allodynia**

The mice were housed in a homemade apparatus with Plexiglas walls and ceiling and a wire mesh floor (3”W x 4”L x 3”H with 0.25” wire mesh). Baseline mechanical thresholds were measured manually using calibrated von Frey filaments (Ugo Basile, Varese, Italy) to stimulate the left hind paw of each mouse. Mechanical thresholds were calculated using the up-down method with four measurements recorded following the first response per mouse on the first day of the experimental timeline before any drug treatment [17; 177].

For CRISPR experiments, mice were returned to the von Frey apparatus after the final injection on Day 3 and allowed to acclimate for 30-60 minutes. Post-CRISPR mechanical threshold measurements were recorded. Mice were then administered 2 mg/kg paclitaxel in a 1:1:4 mixture of Kolliphor EL, absolute ethanol, and USP Saline via the intraperitoneal (IP) route on Days 3, 5, 7, and 9 to induce CIPN. On Day 10, the development of CIPN was confirmed by observing a severe reduction (final threshold <0.5 g) in the mechanical thresholds of each mouse compared to pre-CIPN baseline measurements. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were measured over a three-hour time course.

For siRNA experiments, mice received 2 mg/kg paclitaxel IP for CIPN induction on Days 1, 3, 5, and 7. On Days 5 and 6, the mice were injected with siRNA as described above. On Day 8, the development of CIPN was confirmed by observing a severe reduction (final threshold <0.5 g) in the mechanical thresholds of each mouse compared to pre-CIPN baseline measurements. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were measured over a three-hour time course.

### **Paw Incision Surgery, Mechanical Allodynia, and Recovery**

The mice were measured for mechanical threshold using von Frey filaments as described above. Following baseline measurements, the mice were injected with CRISPR constructs as described above. On Day 9, post-CRISPR mechanical threshold measurements were taken. Immediately following the post-treatment mechanical threshold measurements, plantar paw incision surgery was performed on the left hind paw of each mouse as described in [178]. In brief, anesthesia of each mouse was induced with 3-5% isoflurane in room air and reduced to 1-3% for maintenance for the duration of the surgery. The left hind paw was sterilized by three repetitions of alternating iodine and 70% ethanol in water scrubs with a cotton swab. A 5 mm incision was made through the skin and fascia using a No. 11 scalpel beginning just outside of the foot pads of the hind paw and extending towards the heel to expose the flexor digitorum brevis muscle. Fine tip curved forceps were inserted under the muscle and used to elevate the muscle out of the surgical wound. A separate No.11 scalpel was used to create incisions along the longitudinal axis of the muscle belly. The muscle was replaced and the wound was sutured using 5-0 vicryl sutures. Mice were administered 0.3 mg of gentamicin in USP Saline and 1mL of USP Saline SC and returned to their enclosure for post-operative monitoring. The following day (Day 10), mechanical allodynia was evaluated by a severe reduction (final threshold < 0.5 g) in mechanical thresholds of each mouse compared to pre-surgery baseline measurements. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were recorded over a three-hour time course. Single time point measurements of recovery from surgery-induced mechanical allodynia in the absence of any drug treatment were then recorded on Days 11 and 12.

## **RNA Scope™ Validation of CRISPR Knockdown**

### *Tissue Collection and Preparation*

In separate cohorts from those used in CRISPR behavioral experiments, CRISPR DNA was administered in identical fashion to the behavioral experiments described above. On Day 10 following the beginning of CRISPR delivery, mice were perfused with chilled phosphate-buffered saline (PBS) for 5 minutes followed by chilled 4% paraformaldehyde (PFA) in PBS. Immediately after perfusion with PFA, the spinal column was removed from the mouse, and the spinal cord was ejected by inserting a syringe containing chilled PBS with a 30G needle into the caudal end of the spinal column and applying pressure. Spinal cords were post-fixed in 4% PFA overnight at 4°C. Spinal cords were then dehydrated in 15% sucrose in PBS overnight at 4°C followed by 30% sucrose in PBS overnight at 4°C. After dehydration, spinal cords were flash frozen in optimal cutting temperature compound (OCT) (Fisher Scientific, Cat. No. 23730571) using liquid nitrogen. Tissue was sectioned using an Eprelia HM 525 cryostat at a thickness of 15-20 µm and dry-mounted directly onto SuperFrost Plus™ slides (Fisher Scientific, Cat. No. 1255015). Slides with mounted tissue were left to air dry for at least 2 hours up to overnight at -20°C.

### *RNA Scope™ In-Situ Hybridization*

Prepared slides were allowed to equilibrate to room temperature and then washed with PBS until the excess OCT compound remaining from the mounting process was removed. Slides were baked at 60°C for 30 minutes in a VWR Mini-Incubator (Cat. No. 97025-630) then post-fixed in 4% PFA for 15 minutes at 4°C. Slides then underwent a dehydration series of 50% ethanol, 70% ethanol, and 100% ethanol twice for 5 minutes each at room temperature and then left to air dry.

During the dehydration series, 1X Target Retrieval Buffer was prepared by diluting RNAScope™ 10X Target Retrieval Buffer (ACDBio, Cat. No. 322001, Lot 201955) with deionized water in a beaker. The beaker containing 1X Target Retrieval Buffer and a separate beaker containing deionized water were then placed in a Hamilton Beach Digital Steamer and left to warm. After drying, tissue sections were treated with RNAScope™ H<sub>2</sub>O<sub>2</sub> provided in the RNAScope™ H<sub>2</sub>O<sub>2</sub> and Protease Reagents Kit (ACDBio, Cat. No. 322381, Lot 2023923) at room temperature for 15 minutes and washed in deionized water for 2 minutes. The temperature of the 1X Target Retrieval Buffer was measured to ensure it was within 98-102°C. Slides were then acclimated to the high temperature in the beaker containing deionized water for 10 seconds before being transferred to the Target Retrieval Buffer and incubated for 15 minutes. Slides were washed in deionized water at room temperature for 10 seconds followed by incubation in 100% ethanol for 3 minutes and then left to air dry. After drying, a hydrophobic barrier was drawn around the tissue sections using a Liquid Blocker Super Pap Pen (Electron Microscopy Sciences, Cat. No. 71310) and left to dry overnight at room temperature.

The following day, the incubator was set to 40°C and humidified by lining the bottom with dampened paper towels; paper towels were regularly dampened over the course of tissue treatment to maintain humidity in the incubator. Tissue sections were incubated with Protease III provided with the RNAScope™ H<sub>2</sub>O<sub>2</sub> and Protease Reagents Kit for 30 minutes at 40°C and washed in PBS for 2 minutes. Meanwhile, RNAScope™ 50X Wash Buffer (ACDBio, Cat. No. 320058, Lot 2018155) and RNAScope™ probes for targets of interest were also incubated at 40°C for 15 minutes. Probes used for these experiments were designed by ACDBio to target mouse *Gpr63* (Mm-Gpr63-C2, Cat. No. 319311-C2) and mouse *Gpr153* (Mm-Gpr153-C2, Cat. No. 318101-C2)

and provided at 50X concentrations. After incubation, probes and wash buffer were diluted to 1X using RNAScope™ Probe Diluent (ACDBio, Cat. No. 300041, Lot 2024337) and deionized water, respectively. Following protease treatment of tissue sections, slides were washed with PBS for 2 minutes. Target probes were then hybridized with the tissue sections by adding the prepared 1X RNAScope™ probe mixture and incubating at 40°C for 2 hours. After probe hybridization, slides were washed with 1X Wash Buffer for 2 minutes twice using fresh wash buffer each time. The following signal amplification workflow was performed using reagents from the RNAScope™ Multiplex Fluorescent Detection Kit (ACDBio, Cat. No. 323110, Lot 2023822) unless specified otherwise. Signal amplification took place by treating tissue sections with AMP1, AMP2, and AMP3 solutions for 30, 30, and 15 minutes, respectively, at 40°C with two 2-minute washes using fresh wash buffer in between each amplification step. Slides were then treated with HRP-C2 for 15 minutes at 40°C followed by two 2-minute washes using fresh wash buffer. The fluorescent signal was developed by treating tissue sections with a 1:250 dilution of Opal 620 (Akoya Biosciences, Cat. No. FP1495001KT, Lot 240619012) in RNAScope™ LS Multiplex TSA Buffer (ACDBio, Cat. No. 322810, 2019101) for 30 minutes at 40°C then washed with fresh wash buffer for 2 minutes twice. Tissue sections were then treated with HRP Blocker for 15 minutes and washed for 2 minutes twice with fresh wash buffer. Finally, tissue sections were stained with DAPI for 30 seconds and then immediately mounted using Fluoromount-G™ (Invitrogen, Cat. No. 00-4958-02, Lot E142722) with a No. 1 24X50 mm coverslip (Fisher Scientific, Cat. No. 12541042, Lot 23349) and sealed with clear nail polish (Ted Pella, Inc., Cat. No. 114-7). Fully prepared slides were stored in the dark at 4°C until ready for imaging.

### *Image Acquisition and Analysis*

RNAScope™ slides were imaged on an Olympus Fluoview 1200 laser scanning confocal microscope in the University of Arizona Department of Pharmacology. Images of the spinal dorsal horn were acquired using 20x objectives with filter cube configurations to allow imaging of DAPI, FITC (Opal 520), and Texas Red (Opal 620) emission spectra. Confocal settings were constrained to the following parameters for all channels: HV  $\leq$  750, Gain  $\leq$  5, Offset  $\leq$  50. Image analysis was performed using QuPath software [179]. Regions of interest (ROIs) were drawn around the superficial lamina of the spinal dorsal horn and cell segmentation was performed by the software using the DAPI channel to identify individual nuclei. Cell boundaries were automatically drawn by the software using a set cell diameter of 10  $\mu$ m. QuPath software was then trained to count cells that expressed the GPR target of interest based on signal intensity within the cell boundaries. Cell counts were normalized by recording them as a proportion of the total cell count of the image. A minimum of three technical replicates were obtained for each biological replicate.

### **RT-qPCR Validation of siRNA Knockdown**

#### *Tissue Collection*

In separate cohorts from the siRNA behavioral experiments described above, whole spinal cord tissue for biochemical analysis was collected on Day 4 following the first IT injection. Mice were anesthetized using 5% isoflurane in room air and euthanized by manual cervical dislocation. The spinal column was removed from the mouse and the spinal cord was ejected by inserting a syringe containing chilled PBS with a 30G needle into the caudal end of the spinal column and

applying pressure. Spinal cords were then immediately flash frozen using liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until the experimenter was prepared to perform the RNA isolation.

### *RNA Isolation*

Spinal cord tissue samples were thawed on ice then homogenized manually with 1 mL of TRIzol reagent (Invitrogen, Cat. No. 15596018, Lot 563710) in a Dounce homogenizer. Tissue homogenates were then transferred to 1.5 mL Eppendorf tubes and incubated on ice for at least 5 minutes or overnight at  $4^{\circ}\text{C}$ . Tissue homogenates were then combined with 200  $\mu\text{L}$  of chloroform, mixed by shaking, and then incubated on ice for 3 minutes. Samples were centrifuged for 15 minutes at 12,000g at  $4^{\circ}\text{C}$ . The upper aqueous layer was carefully transferred to a separate 1.5 mL Eppendorf tube using a micropipette and the remaining layers were discarded. The aqueous layer was then combined with 500  $\mu\text{L}$  of isopropanol and incubated for 30 minutes at  $4^{\circ}\text{C}$ . This mixture was then centrifuged for 10 minutes at 12,000g at  $4^{\circ}\text{C}$  to yield a white RNA pellet. RNA pellets were isolated by carefully aspirating the supernatant and resuspended in 1mL of 75% ethanol in nuclease-free water. The resuspended pellet was then centrifuged for 5 minutes at 7,500g at  $4^{\circ}\text{C}$ , the supernatant was carefully aspirated again, and the pellet was air dried for 10 minutes. After drying, the RNA pellet was dissolved in 50  $\mu\text{L}$  of nuclease-free water and incubated in a heating block at  $55\text{-}60^{\circ}\text{C}$  for 15 minutes. RNA concentration and quality was assessed using a NanoDropND-1000 spectrophotometer (NanoDrop Technologies) and stored at  $-80^{\circ}\text{C}$  until the experimenter was prepared for the reverse transcription reaction.

### *Reverse Transcription Reaction for cDNA Generation*

RNA samples and the High Capacity RNA-to-cDNA<sup>TM</sup> Kit (Applied Biosystems, Cat. No. 4387406, Lot 2945899) were thawed on ice. The reverse transcription reaction mixture for each sample was composed of the following: 10  $\mu$ L of 2X RT Buffer Mix, 1  $\mu$ L of 20X RT Enzyme Mix, 1  $\mu$ g of RNA (volume calculated based on sample concentration), and nuclease-free water in a quantity sufficient to dilute the total volume to 20  $\mu$ L. Reverse transcription was performed using a BioRad C1000 Touch Thermal Cycler using the following parameters: 37°C for 60 minutes, 95°C for 5 minutes, 4°C for at least 10 minutes. Following the final 4°C cooling step, samples were removed from the thermal cycler and stored at 4°C or -20°C long term until the experimenter was prepared for real-time quantitative PCR analysis.

### *Real-Time Quantitative PCR and Quantification*

Real-time quantitative PCR was performed using a 96-well plate with each well containing a mixture of the following components: 0.3  $\mu$ L of the target forward primer, 0.3  $\mu$ L of the target reverse primer, 1  $\mu$ L of cDNA, 10.9  $\mu$ L of nuclease-free water, and 12.5  $\mu$ L of PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master Mix (Applied Biosystems, Cat. No. A25742, Lot 3089500). Each reaction was run in triplicate on a BioRad C1000 Touch Thermal Cycler using the following parameters: 95°C for 10 minutes followed by 45 cycles of i) 95°C for 15 seconds, ii) 60°C for 1 minute followed by a final step at 72°C for 10 minutes. Relative RNA abundance was calculated by  $1/2^{\Delta\text{CycDiff}}$  of the GPR target of interest compared to internal GAPDH control and normalized to the NC siRNA treated control group from the same plate. Custom forward and reverse primers for mouse *Gpr63* and *Gpr153* were created by Integrated DNA Technologies (sequence information

below). GAPDH primers were also obtained from IDT; sequence information for these can be found in [180].

### *Primers*

Mouse *Gpr63* Forward, Reference #460670820: (5' – TGG CTA CTC TGG CTC TGC TA – 3')

Mouse *Gpr153* Reverse Reference #460670821: (5' – CGA TGT TCC CCA CAC ACG TA – 3')

Mouse *Gpr153* Forward Reference #460670822: (5' – GCA GCA TTA GCA GCT TTC TGA – 3')

Mouse *Gpr153* Reverse Reference #460670823: (5' – TCC ACC ATC CAT TCA CCC CT – 3')

### **Statistical Analysis**

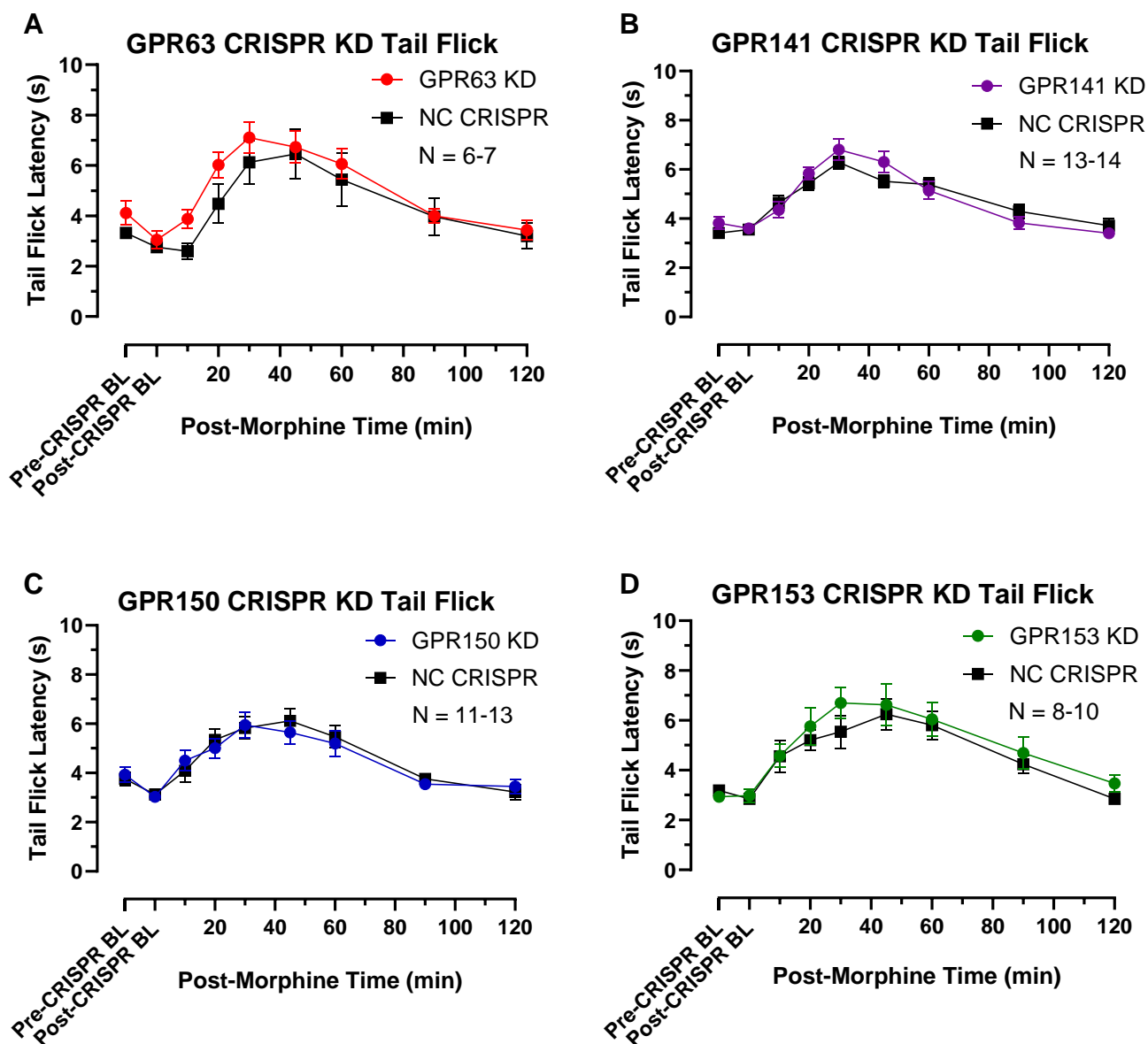
All data were reported as the mean  $\pm$  SEM. RT-qPCR data was normalized to an internal GAPDH control and RNAScope<sup>TM</sup> cell counts were normalized to the total cell counts (as determined from DAPI stain) from their respective ROIs. Behavioral data were reported raw with no maximum possible effect or other normalization applied. Statistical comparisons between groups were performed by Student's t-test (unpaired, two-tailed) for RNAScope<sup>TM</sup> and RT-qPCR experiments or two-way analysis of variance (ANOVA) with Šídák's post hoc test for behavioral experiments. In all cases, statistical significance was defined as  $p < 0.05$ . All graphing and statistical analyses were performed using GraphPad Prism 9 software (San Diego, CA).

## 2.3: Results

### 2.3.1: CRISPR Knockdown of All Orphan GPCR Candidates Has no Effect on Acute Thermal Nociception or Opioid-Induced Antinociception *In Vivo*

To our current knowledge, none of the targets chosen for this study have been investigated in the context of nociception or interactions with opioid-induced analgesia, thus making this endeavor a venture into uncharted territory. From an exploratory scientific perspective, this paradoxically presents both significant freedoms and severe limitations in how we can initiate experiments with these targets. It is especially challenging to develop informed hypotheses about the functions of our targets given the general lack of relevant literature available. Our lab is well equipped and has established an extensive record in investigating novel analgesic drug targets through behavioral assays in mice [15-18]. This experience allowed for speculation of multiple potential outcomes: (1) knockdown of our targets could have direct effects on nociception, either increasing or decreasing baseline pain-like responses, and/or (2) knockdown of our targets could have positive or negative effects on opioid-induced antinociception. Having multiple targets also introduced the possibility of opposing results that could lead to more developed hypotheses regarding each receptor's possible function in the spinal cord. The spinal cord is of particular interest in this work and our lab broadly as it is the first processing center of nociceptive inputs in the CNS as demonstrated by the neurocircuitry of pain and the mechanisms of action of current pharmacotherapies introduced in **Chapter 1**. Nonetheless, we began with the simplest and most common behavioral assay at our disposal: the hot water tail flick.

We performed CRISPR knockdowns of all oGPCR targets within the mouse spinal cord along with age-matched negative controls (NC) and found that there was no significant effect of any knockdown on tail flick latencies compared to the NC groups in the absence of a 3.2 mg/kg screening dose of morphine (**Fig. 2.2**). Furthermore, no oGPCR knockdown altered the response to morphine over a two-hour time course (**Fig. 2.2**). These findings could be interpreted as none of our targets having impacts on acute nociceptive pain states. This outcome may be favorable since as described in **Chapter 1** acute pain is not a negative outcome that needs to be mitigated but rather necessary for survival. Furthermore, the use of opioids for the treatment of acute nociceptive pain states is generally unnecessary. This also comes with the caveat that the tail flick assay is only evaluating the thermal modality of pain whereas mechanical nociception must be addressed using other models.



**Figure 2.2: CRISPR knockdown of target oGPCRs does not affect thermal nociception or opioid-induced antinociception in a mouse model of acute nociceptive pain.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63, (B) GPR141, (C) GPR150, or (D) GPR153 or a negative control (NC) CRISPR construct in the spinal cord via IT injection as described in the Methods. Pre-CRISPR baseline tail flick latencies were measured

immediately prior to the first IT injection, and post-CRISPR baseline tail flick latencies were measured on Day 10 following the first IT injection (52°C, 10 sec cutoff). Animals were then administered a 3.2 mg/kg screening dose of morphine SC and tail flick latencies were assessed over a two-hour time course. There was no statistically significant difference between the oGPCR knockdown treated groups compared to the matched NC treated groups by two-way ANOVA with Šídák's post hoc test. Data reported as mean  $\pm$  SEM.

### 2.3.2: CRISPR Knockdown of GPR63 and GPR153 Ablates Opioid-Induced Antinociception in a Mouse Model of CIPN-Induced Mechanical Allodynia While Having no Impact on Baseline Mechanical Nociception

Having found no effect of CRISPR knockdown of the four candidate oGPCRs in acute thermal nociception, the next most common pain model established in our lab is CIPN-induced mechanical allodynia. Paclitaxel is a chemotherapy drug commonly used clinically for the treatment of various cancer types [181; 182]. Like many other chemotherapy drugs, reports of numbness or tingling in the extremities is often reported by cancer patients undergoing chemotherapy with paclitaxel as a side effect. This outcome results from paclitaxel-induced neurotoxicity by a mechanism of action in stabilizing microtubules to mitigate cancer cell proliferation, which also contributes to axonal degeneration of peripheral neurons that require microtubule dynamics for maintenance [28].

In identical fashion to the previous tail flick experiments, spinal cord CRISPR knockdown of all four oGPCR targets was performed along with age-matched cohorts of NC treated mice for each group and CIPN induced using 2 mg/kg paclitaxel as in our previous work [17; 95]. We observed that knockdowns of both GPR63 and GPR153 both completely ablated the antinociceptive effects of the 3.2 mg/kg morphine used to treat the paclitaxel-induced mechanical allodynia whereas GPR141 and GPR150 knockdown mice demonstrated responses to morphine comparable to their NC counterparts (**Fig. 2.3**). This result is the first implicating GPR63 and GPR153 as potential modulators of the development of pathological pain states and/or opioid-induced antinociception in a pathological pain state. This result led to the development of two potential hypotheses regarding how this effect is mediated biologically: (1) the removal of GPR63

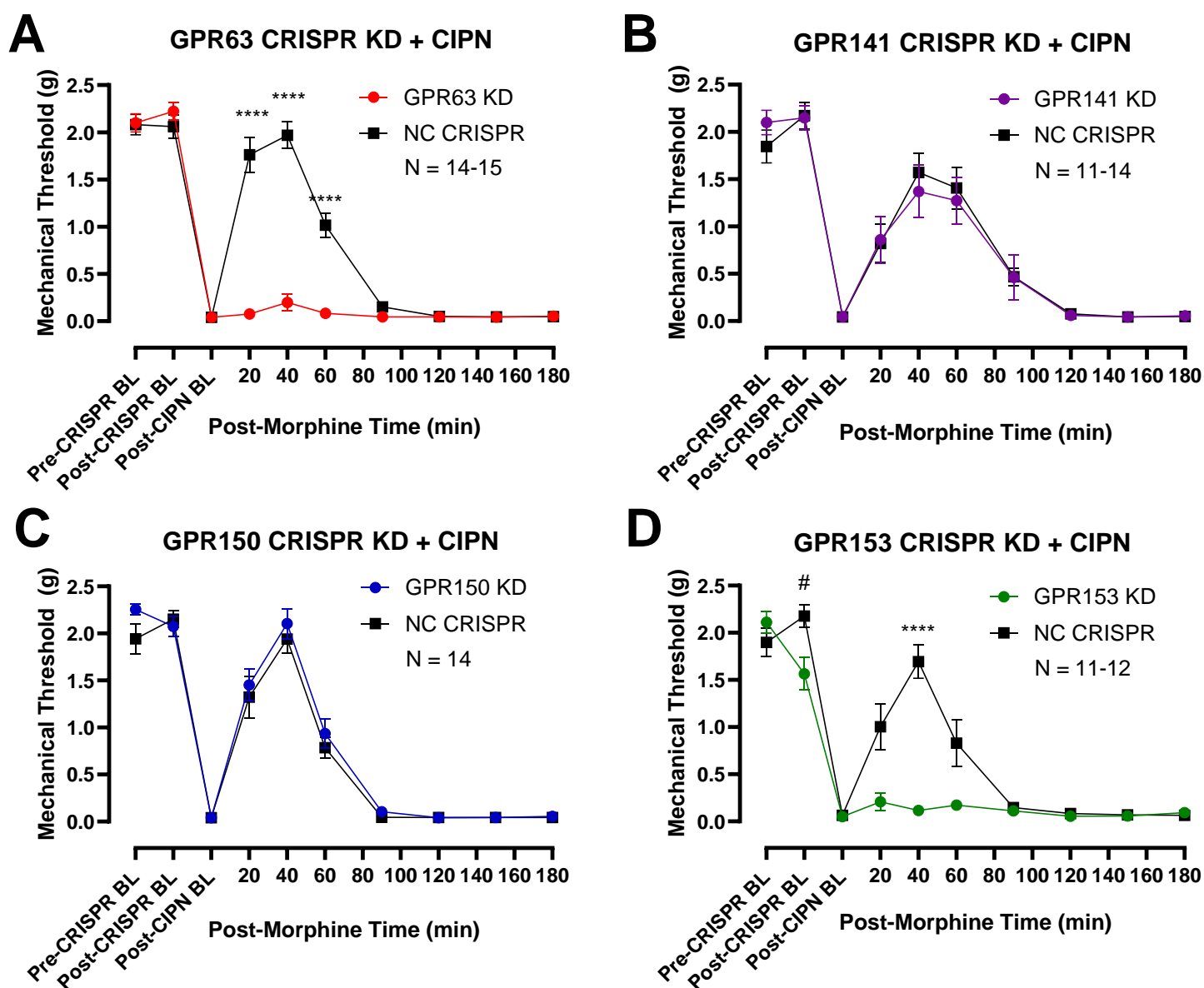
and GPR153 dysregulates opioid-induced antinociception by some interaction with the nociceptive circuit that results in the administered dose being ineffective or (2) the removal of GPR63 and GPR153 worsens the underlying pathology of CIPN-induced mechanical allodynia to a degree such that it renders the administered dose ineffective. Furthermore, it was noted that there was a significant reduction in mechanical threshold in GPR153 CRISPR treated mice when comparing the pre- and post-CRISPR baseline measurements before CIPN was induced, and this may also implicate GPR153 in acute mechanical nociception (**Fig. 2.3D**).

To address the secondary observation of the GPR153 CRISPR dataset in **Figure 2.3D** and the latter of the two hypotheses generated by our original results, a modified von Frey experiment was performed. CRISPR knockdown of GPR63 and GPR153 and CIPN induction were all performed identically to the experiments described in **Figure 2.3**, but mice were stimulated repeatedly with the same von Frey filament (0.4 g) for a total of ten trials at the pre- and post-CRISPR and post-CIPN time points to measure response frequency rather than using a full calibrated set of filaments to measure mechanical thresholds. This approach offers higher sensitivity to detect changes in nociceptive baselines. By this method, we observed that knockdown of neither target oGPCR had any impact on response frequency between the pre- and post-CRISPR time points, suggesting that there is no direct effect on acute mechanical nociception (**Fig. 2.4**). We also found that CRISPR knockdown of both targets had no effect on the severity of CIPN-induced mechanical allodynia resulting in failure to support hypothesis (2) thus far (**Fig. 2.4**).

The incredibly novel nature of the findings presented in **Figure 2.3** also demonstrated the need for thorough validation to minimize the possibility that off-target effects caused by CRISPR

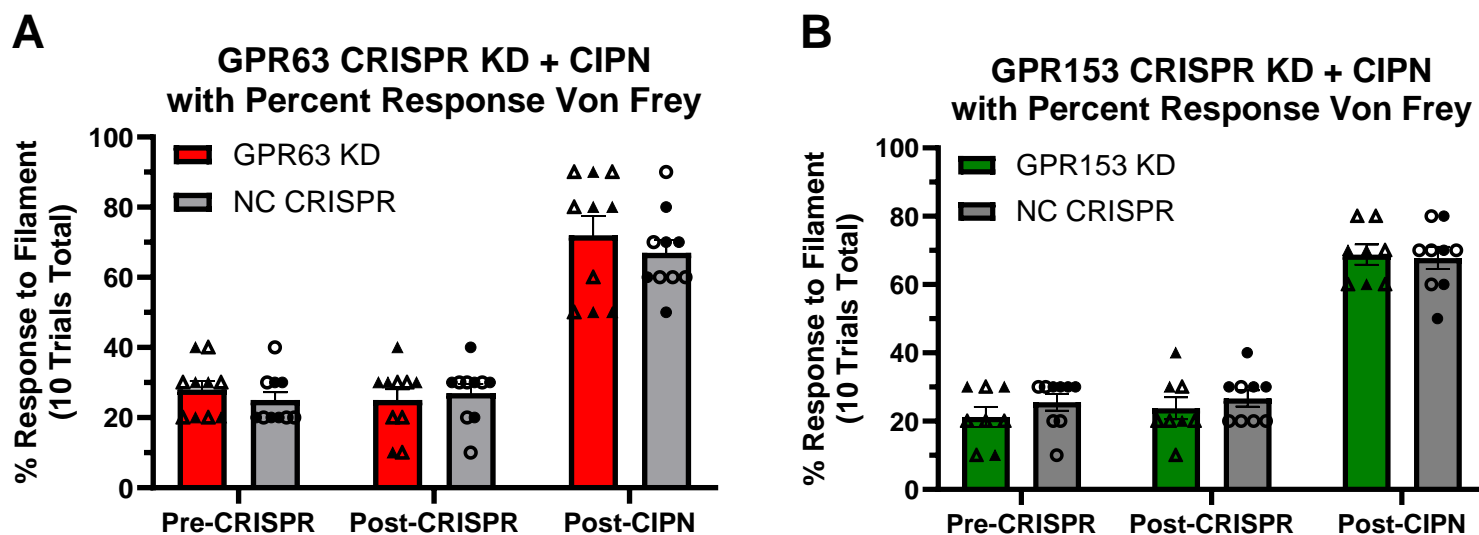
knockdown of GPR63 and GPR153 were responsible for the behavioral outcomes we observed. A different knockdown approach utilizing siRNA targeting GPR63 and GPR153 was performed alongside a similar CIPN induction time course to replicate the behavioral results of **Figure 2.3A and 2.3D**. This approach targets these oGPCRs after transcription rather than directly modifying the genome, and is thus unlikely to share off-target interactions with our CRISPR approach. Fortunately, we observed that siRNA knockdown of both GPR63 and GPR153 also ablated opioid-induced antinociception in a CIPN mouse model (**Fig. 2.5A, 2.5C**) and confirmed our previous findings. This method of receptor knockdown was further validated by RT-qPCR using spinal cord tissue collected from behavioral testing subjects which demonstrated approximately 50% knockdown efficiency (**Fig. 2.5B, 2.5D**). The specificity of our CRISPR construct was also evaluated visually using RNAScope<sup>TM</sup> *in situ* hybridization targeting GPR63 and GPR153 mRNA transcripts in the spinal dorsal horn (**Fig. 2.6**). By this method, our CRISPR delivery demonstrated high efficacy in reducing GPR63 and GPR153 mRNA expression compared to NC CRISPR treated mice (**Fig. 2.6B, 2.6D**). These validated experiments thus strongly support a specific role for GPR63 and GPR153 in regulating opioid antinociception during pathological pain.

Thus far, the combination of behavioral results across the tail flick and CIPN von Frey assays followed by validation of our CRISPR methods justified continuation of the behavioral screen for GPR63 and GPR153 only. This decision allowed for a more focused approach in exploring the mechanism of action of GPR63/153 during pathological pain in further studies.



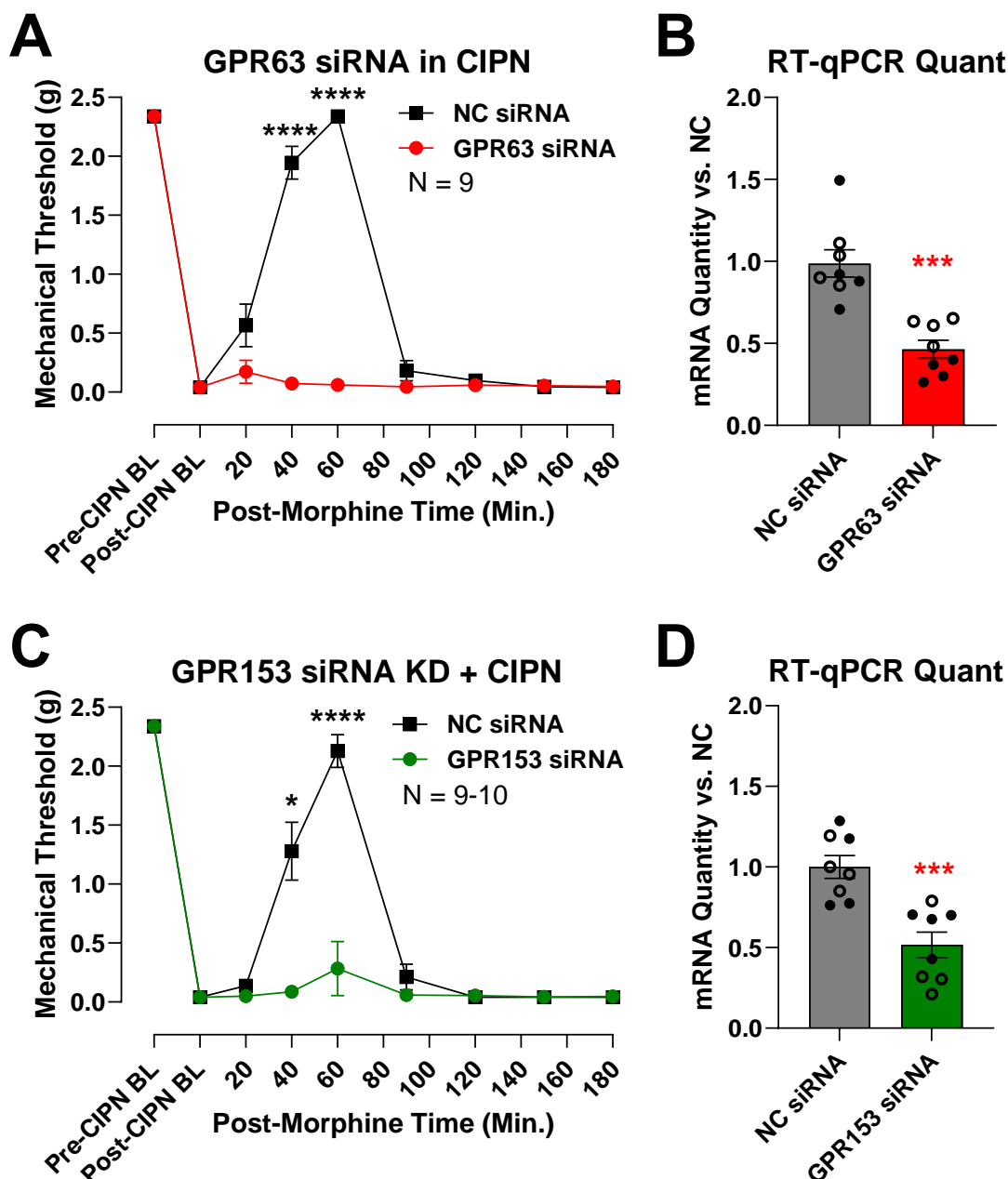
**Figure 2.3: CRISPR knockdown of GPR63 and GPR153 completely ablates opioid-induced antinociception in a mouse model of CIPN.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63, (B) GPR141, (C) GPR150, or (D) GPR153 or a negative control (NC) CRISPR construct in the spinal cord via IT injection as described in the Methods. Pre-CRISPR mechanical threshold baselines were measured using calibrated von Frey

filaments immediately prior to the first IT injection, and post-CRISPR mechanical thresholds were measured after the final IT injection after animals were allowed to reacclimate to the von Frey testing apparatus. Immediately following the post-CRISPR baseline measurement, mice received 2 mg/kg paclitaxel IP on Days 3, 5, 7, and 9. On Day 10, the development of mechanical allodynia by CIPN was confirmed by a dramatic decrease in mechanical threshold. Mice were treated with 3.2 mg/kg morphine SC and mechanical thresholds were evaluated over a three-hour time course. Data reported as mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$  vs. NC CRISPR group at the same time point by repeated measures two-way ANOVA with Šídák's post hoc test. # $p < 0.05$  vs. Pre-CRISPR BL within group. CRISPR knockdown of **(A)** GPR63 and **(D)** GPR153 abolished the antinociceptive effects of morphine in this model and knockdown of **(D)** GPR153 alone produced a statistically significant reduction in mechanical threshold after CRISPR administration but before induction of CIPN, potentially linking it to baseline mechanical nociception as well.



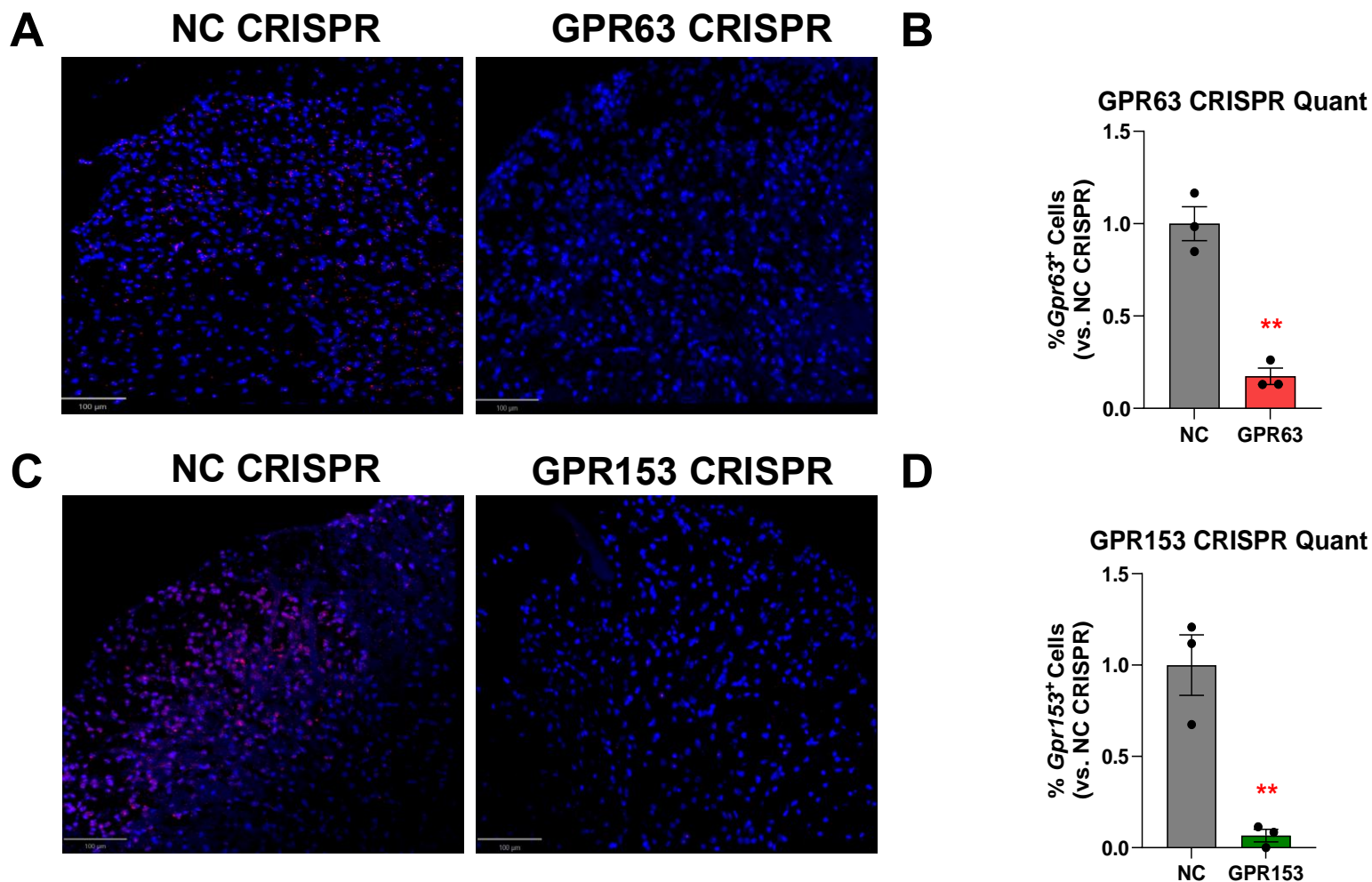
**Figure 2.4: CRISPR knockdown of GPR63 and GPR153 does not affect baseline mechanical nociception or the severity of mechanical allodynia in a mouse model of CIPN.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (**A**) GPR63 or (**B**) GPR153 or a negative control (NC) CRISPR construct in the spinal cord via IT injection as described in the Methods. A modified von Frey protocol was used in which the left hind paw of the mouse was stimulated using the same filament (0.4 g) ten times. Responses were recorded as a percentage out of the ten total trials. Pre-CRISPR baseline measurements were taken prior to the first IT injection on Day 1 and post-CRISPR baseline measurements were taken after the final IT injection and allowing mice to reacclimate to the von Frey apparatus on Day 3. Neither knockdown of (**A**) GPR63 or (**B**) GPR153 had a significant effect on acute mechanical nociception. CIPN was then induced by administering 2 mg/kg paclitaxel IP on Days 3, 5, 7, and 9. Development of mechanical allodynia by CIPN was demonstrated by an overall increase in response frequency in proportion to the total stimulus count, but knockdown of neither (**A**) GPR63 or (**B**) GPR153 had any effect on the severity of mechanical allodynia. Data reported as mean  $\pm$  SEM with individual

data points. All data was analyzed by repeated measures two-way ANOVA ( $p > 0.05$ ). Open symbols represent female mice and closed symbols represent male mice.



**Figure 2.5: Validation of GPR63 and GPR153 CRISPR results by siRNA knockdown in a mouse model of CIPN.** Baseline mechanical thresholds in male and female CD-1 mice were measured using a set of calibrated von Frey filaments. CIPN was then induced by administering 2 mg/kg paclitaxel on Days 1, 3, 5, and 7. On Days 5 and 6, mice were injected with 1  $\mu$ g of siRNA targeting either (A) GPR63 or (C) GPR153 or a negative control (NC) siRNA that had no gene target. The development of mechanical allodynia by CIPN was confirmed by measuring

mechanical thresholds on Day 8. Mice were then treated with a 3.2 mg/kg screening dose of morphine and mechanical thresholds were assessed over a three-hour time course. Knockdown of both (A) GPR63 and (C) GPR153 by siRNA yielded the same behavioral results observed in **Figure 5** thus validating our CRISPR knockdown. The effectiveness of the siRNA knockdown for GPR63 and GPR153 was evaluated by RT-qPCR in (B) and (D), respectively, indicating a ~50% knockdown efficiency. Data reported as mean  $\pm$  SEM. Behavioral data were analyzed by repeated measures two-way ANOVA with Šídák's post hoc test. \*,\*\*\*\*p < 0.05, 0.0001 vs same time point siRNA group. RT-qPCR data was analyzed by an unpaired 2-tailed Student's *t*-test. \*\*\*p < 0.01, 0.001. Open symbols represent female mice and closed symbols represent male mice.



**Figure 2.6: RNAScope validation of CRISPR Knockdown of GPR63 and GPR153 in the mouse spinal dorsal horn.** Female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63 or (C) GPR153 or a negative control (NC) CRISPR construct in matching cohorts in the spinal cord via IT injection as described in the Methods. The effectiveness of CRISPR knockdown of each target compared to their NC matched cohorts was visualized by RNAScope™ *in situ* hybridization as described in **Section 2.2: Materials and Methods** and imaged at 20x magnification on an Olympus FV1200 laser-scanning confocal microscope. Scale

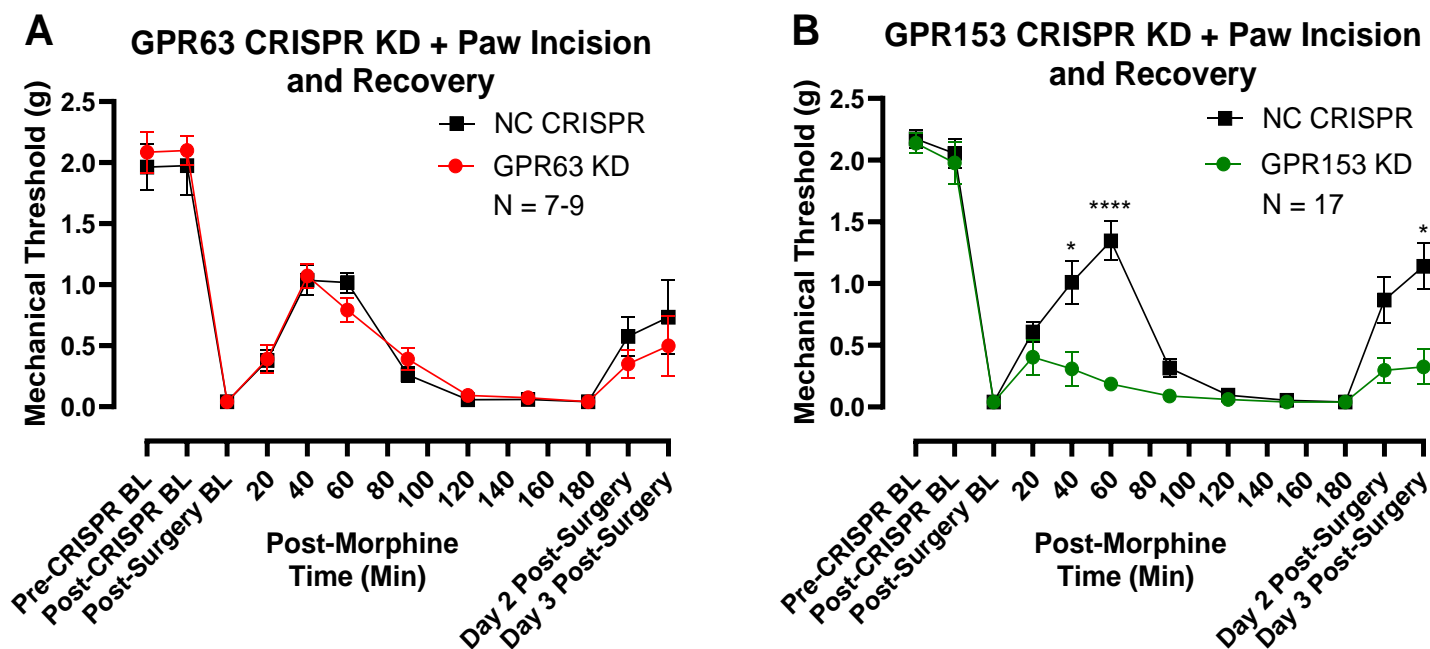
bars represent 100  $\mu\text{m}$ . Quantification of RNAScope<sup>TM</sup> results was performed by automated cell counting using QuPath bioimage analysis software. Knockdown of **(B)** GPR63 and **(D)** GPR153 was found to be effective by our CRISPR method, demonstrating a significant decrease in target oGPCR expression compared to matched controls. Data reported as mean  $\pm$  SEM. Data was analyzed by unpaired 2-tailed Student's *t* test. \*\* $p < 0.01$ .

### 2.3.3: CRISPR Knockdown of GPR153 but not GPR63 Abolishes Opioid-Induced Antinociception and Impairs Recovery in a Mouse Model of Post-Operative Pain

Our results so far suggested that GPR63 and GPR153 regulate opioid antinociception in pathological but not nociceptive/uninjured pain. To further explore this hypothesis, we tested GPR63/153 regulation of a paw incision post-operative pain model. Post-operative pain is unique in that the mechanisms in which it is nociceptive are initially normal acute responses caused by the incision and resulting inflammation, but this can become maladaptive. Clinically, chronic post-surgical pain (CPSP) stems from acute pain inherent with surgical wounds failing to resolve after healing, but the underlying mechanisms leading to the transition from acute post-surgical pain to CPSP are complex and not fully understood [8; 183]. The importance of considering CPSP as a pathological pain state is crucial, however, considering that CPSP is estimated to develop in 10% of all surgical cases worldwide [183]. Unlike the previously evaluated pain models, post-operative pain is also an attractive method for this screen in the context of pathological pain as it sits between having features of both acute and chronic pathological pain states while also allowing us to monitor recovery or the lack thereof.

In this work, post-operative pain was modeled using the plantar paw incision surgical procedure as described in [178]. CRISPR knockdown of GPR63 and GPR153 was performed identically to the previous tail flick and CIPN von Frey experiments. Mechanical allodynia induced by planter paw incision was treated with a 3.2 mg/kg dose of morphine SC. Mice that received CRISPR knockdown of GPR63 responded normally to the administered morphine and experienced comparable opioid-induced antinociception to the matched NC group (**Fig. 2.7A**). By contrast, GPR153 knockdown again abolished the antinociceptive effects of morphine (**Fig. 2.7B**). To

investigate the ability for mice to recover from post-surgical mechanical allodynia, single time point measurements in the absence of morphine were also taken for two consecutive days following the full von Frey time course. GPR63 CRISPR knockdown continued to have no effect with mice recovering at a similar rate to the NC group (**Fig. 2.7A**). Interestingly, GPR153 CRISPR treated mice showed persistent mechanical allodynia compared to their NC treated counterparts (**Fig. 2.7B**). This result may also be suggestive of a potential role for GPR153 in the recovery and resolution of post-surgical pain.



**Figure 2.7: CRISPR knockdown of GPR153 but not GPR63 mitigates opioid-induced analgesia and impairs recovery from mechanical allodynia in a mouse model of post-operative pain.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63 or (B) GPR153 or a negative control (NC) CRISPR construct in the spinal cord via IT injection as described in the Methods. Pre-CRISPR baseline mechanical threshold measurements were taken prior to the first IT injection on Day 1 and post-CRISPR baseline measurements were taken on Day 9 immediately prior to the post-surgical pain model. On Day 9, mice underwent plantar paw incision surgery of the left hind paw and were allowed to recover overnight. The following day (Day 10), mechanical allodynia caused by the surgical wound was confirmed via von Frey. Mice were then treated with 3.2 mg/kg morphine SC and mechanical threshold was measured over a three-hour time course. Single time point measurements in the absence of morphine were then taken on Days 11 and 12 to measure recovery from post-surgical

mechanical allodynia. CRISPR knockdown of **(A)** GPR63 had no impact on opioid-induced analgesia or recovery from surgery whereas **(B)** GPR153 significantly reduced opioid-induced analgesia and diminished recovery from mechanical allodynia two and three days following the surgical procedure. Data were analyzed by two-way repeated measures ANOVA with Šídák's post hoc test. \*,\*\*\*\*p < 0.05, 0.0001 vs same time point group.

## 2.4: Conclusion

Here we have performed a behavioral screen of four orphan GPCRs with no currently known ligands or functions within the CNS. In the context of pathological pain states, GPR63 and GPR153 have proven to be viable candidates for further investigation following the discovery that their knockdown seemingly prevents opioid-induced antinociception in CIPN and knockdown of the latter prevents opioid-induced antinociception and impairs recovery in post-surgical pain. None of our initial targets demonstrated any impact on nociceptive/uninjured thermal or mechanical nociception, suggesting they may be selective for pathological pain. The current findings suggest that GPR63 and GPR153 are responsible for modulating opioid antinociception but only in pathological pain states, and not by modulating the underlying pathology of the pain state itself. Meanwhile they appear to have no significant role in acute/uninjured nociception or opioid antinociception. This outcome may be favorable, however, as it was described in **Chapter 1** that acute pain is not a negative outcome that needs to be mitigated but is rather necessary for survival, and the use of opioids for the treatment of acute/nociceptive/uninjured pain states is generally unnecessary. Other non-opioid targets for pain management have encountered challenges in their drug development cycles for this very reason, proving to be effective in generating antinociception but also reducing sensitivity to acutely painful stimuli and increasing the risk of injury such as the case for antagonists of TRPV1 [184].

It is important to note that this screen is not fully comprehensive. Presently, our only model of testing nociceptive pain was the hot water tail flick assay which restricts our conclusions on the effect of oGPCR knockdown specifically to the modality of thermal nociception. Furthermore, the argument can be made that the tail flick assay itself is not fully adequate for assessing pain-like

behaviors because it is mediated by a spinal nociceptive reflex and does not require higher order processing by the test subject in order to yield a response [185]. Other behavioral assays that assess thermal nociception such as the Hargreaves or hot plate tests could be useful in interrogating the effects of target receptor knockdown further as the nocifensive responses to heat stimuli in these assays are considered to incorporate supraspinal pathways [177]. Incorporating assays to test other modalities of nociceptive pain such as the Randall-Selitto test for mechanical nociception may also be able to address limitations in our von Frey approaches. The pain models listed here also did not investigate the impact of oGPCR knockdown on inflammatory pain conditions that can be modeled by intraplantar LPS or CFA administration followed by von Frey assessment. Lastly, all of the measures of pain-like behavior performed in this chapter are stimulus-evoked. It is important to consider that spontaneous pain in the absence of a noxious stimulus and impairments to quality of life are notable in pathological pain sufferers, and pathological pain can yield similar outcomes in rodents that are measurable through behaviors such as grimacing, burrowing, or weight bearing [177; 186-189]. Although our behavioral screening efforts have produced both positive and negative results that provide some insights into the functions of the oGPCRs investigated here, there remains opportunities for further study of their impacts in pain-like behaviors.

In consideration of the lack of evidence supporting involvement in acute nociception, we can currently speculate that the biological mechanism by which GPR63 and GPR153 knockdown influences opioid-induced analgesia in pathological pain may be mediated by a glial cell-based mechanism rather than directly influencing nociceptors. Glial cells such as microglia and astrocytes within the spinal cord have been extensively implicated in the development and maintenance of chronic and pathological pain, and both GPR63 and GPR153 have been linked to

astrocytes and microglia, respectively, via expression mapping of numerous CNS oGPCRs [143; 190-194]. Additionally, these glial cells have been implicated in affecting the antinociceptive capacity of opioids and the development of adverse side effects (e.g., tolerance, hyperalgesia) [10; 195-197]. As such, the remainder of this work will examine the remaining candidate oGPCRs in relation to astrocytes and microglia in order to gain further insights regarding the potential biological mechanisms that contribute to the observed behavioral outcomes presented thus far.

**Chapter 3: Investigating the Role of Spinal Cord GPR63 and  
GPR153 in Pathological Pain States via Microglia and  
Astrocytes**

### 3.1: Introduction

As mentioned briefly in the previous chapter, microglia and astrocytes in the CNS are known to be key players in the development and maintenance of chronic and pathological pain conditions while also having roles in the modulation of opioid pain relief and side effects. This context in combination with our inability to show any effect of GPR63 and GPR153 knockdown on opioid-induced antinociception or baseline nociception in healthy animals has led us to focus our research efforts on these targets in glial cell types. Glial cells play numerous roles in the CNS and in the periphery. Microglia have been linked to important functions such as facilitating apoptosis, synaptic pruning during development, and even adult neurogenesis [198-200]. They also act as the resident immune cells of the CNS through their phagocytic activity and production of both pro- and anti-inflammatory cytokines in response to tissue injury or neurodegenerative diseases [200-203]. Astrocytes are well recognized for establishing the blood-brain barrier protecting the CNS, supporting neuronal metabolism, and facilitating synaptic communication [204-209]. This cell type has also received significant interest as a therapeutic target in CNS disease states through their proven roles in neural repair and neuroinflammatory processes [210; 211]. Analysis of spinal cord cell populations across species have demonstrated that glial cells outnumber neurons in the gray matter of the spinal cord, especially in humans, thus lending credence to the pursuit of analgesic targets through these cell types with high potential for clinical significance [212; 213].

Like oGPCRs, glial cells have also been hot topics of investigation in the search for novel analgesic drug targets to combat chronic and pathological pain and the ongoing opioid epidemic [214; 215]. Microglia express many targets currently studied for the development of analgesics

such as the purinergic receptor P2X<sub>4</sub>R, toll-like receptor 4 (TLR4), and p38 mitogen-activated protein kinase (p38 MAPK), a signaling molecule downstream of several receptors including P2X<sub>4</sub>R and TLR4 and which is linked to inflammatory pathways [215-218]. Following nerve injury, microglia will also express numerous pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) which trigger a cascade of neuroinflammatory processes among neighboring microglia and are capable of sensitizing nociceptors [40; 203; 219; 220]. Neuroinflammation itself is heavily linked to pathological pain conditions and the roles of microglia in developing a neuroinflammatory environment quickly in response to nerve injury has led to them being viewed as crucial mediators in the onset of pathological pain [219]. Microglia have also been demonstrated to affect the resolution of pathological pain states if they present an anti-inflammatory phenotype [221; 222]. Depletion and repopulation of microglia in an injury model also demonstrates that repopulated microglia have different transcriptomic profiles compared to those present at the onset of pathological pain which may contribute to pain resolution [223].

By contrast, astrocytes have proven roles in the long-term maintenance and perpetuation of pathological pain states with astrogliosis having the capability of persisting months beyond nerve injury [214; 224]. Astrogliosis can be triggered by the release of inflammatory cytokines from neighboring microglia, and astrocytes themselves also showcase elevated expression of these cytokines in pathological pain states [224-226]. This is in line with some findings that microglial activation precedes astrogliosis in cases of CIPN and chronic post-surgical pain [225; 227; 228]. Reactive astrocytes themselves demonstrate reduced efficiency at performing their

aforementioned functions which has the potential to compound damage or hypersensitivity to nociceptive neurons [224; 229].

Both microglia and astrocytes have been implicated in the underlying pathologies of the pain models discussed in this work. Inflammatory cytokines produced by glial cells reduce inhibitory modulation of the spinal pain circuit resulting in increased nociceptive signaling [230; 231]. CIPN itself is heavily characterized by neuroinflammation [10; 232; 233]. Interestingly, models of CIPN using different chemotherapy agents may induce astrocyte or microglia activation to varying degrees which further complicate the matter [234; 235]. Models of post-surgical pain also demonstrate similar cascades of neuroinflammation in the spinal dorsal horn through the activation of and crosstalk between microglia and astrocytes [227; 236].

Glial cells also demonstrate interactions with the endogenous opioid system in rodent models despite the apparent absence of MOR expression in both astrocytes and microglia of the rat spinal cord ([237], although this conclusion is controversial). It has been reported that morphine can activate TLR4 on microglia and exacerbate neuroinflammation to counter opioid analgesia by increasing glutamatergic signaling onto second order nociceptive neurons [238-240]. These outcomes are considered to be contributors to opioid-induced hyperalgesia (OIH) and the development of opioid tolerance with chronic use [195; 240]. Astrocytes also play roles in generating OIH and maladaptive synaptic plasticity through interactions with neurons in the spinal dorsal horn [197]. All of the above factors together suggest that GPR63 and GPR153 could carry out the observed effects on pathological pain in **Chapter 2** via microglia and/or astrocytes which is further supported by mouse transcriptomic data linking GPR63 and GPR153 expression to astrocytes and microglia, respectively [143].

## 3.2: Materials and Methods

### **Drugs**

Morphine sulfate pentahydrate was obtained through the National Institute on Drug Abuse Drug Supply Program and distributed through the Research Triangle Institute. Paclitaxel (Cat. No. AAJ6273403, Lot Q25K025) and minocycline (Cat. No. J66429, Lot U24E007) were purchased from Fisher Scientific (Waltham, MA). Gentamicin sulfate (Cat. No. 1098195) was purchased from Henry Schein (Melville, NY). Morphine and paclitaxel were crystalline white solids and stored by manufacturer's recommendations (morphine at 20-25 °C; paclitaxel at -20 °C). Gentamicin was received in 2 mL vials at a concentration of 40mg/mL in USP Saline. Morphine was prepared fresh for every experiment in USP Saline. Paclitaxel was prepared as a stock solution in a 1:1 mixture of Kolliphor EL (Fisher Scientific, Cat. No. 50-165-7076) and absolute ethanol (Fisher Scientific, Cat. No. BP28184) and stored at -20 °C. Gentamicin was prepared as a stock solution in USP Saline and stored at -20 °C. Minocycline was prepared fresh for every experiment in USP Sterile Water.

### **Animals**

Male and female CD-1 mice aged 5-8 weeks from Charles River Laboratories (Wilmington, MA) were used for all experiments. CD-1 [(also known as Institute for Cancer Research (ICR)] mice are commonly used in opioid research and in our previous work as an outbred mouse strain with strong responses to opioid drugs [15-18]. Male and female mice were used in approximately equal

numbers for all behavioral experiments. No sex differences were observed in behavioral experiments; thus, all data shown for behavioral experiments was pooled from male and female cohorts. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) – accredited vivarium at the University of Arizona where they were acclimated for at least five days after shipment before being used for experiments. Twelve-hour light and dark cycles were maintained with food (standard chow) and water available *ad libitum*. Mice were housed with no more than five per cage. Animal monitoring was provided daily, including after surgical procedures, by trained veterinary staff. All experiments were performed in accordance with Institutional Animal Care and Use (IACUC) – approved protocols at the University of Arizona and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### **CRISPR Knockdown**

All-in-one predesigned CRISPR constructs containing sgRNA and Cas9 expression cassettes were obtained from Genecopoeia (Rockville, MD). Knockdown constructs specifically affecting microglia were designed to target mouse *Gpr63* (Cat. No. CS-MCP002166-CG12-1-01-10) or mouse *Gpr153* (Cat. No. CS-MCP002167-CG12-1-01-10) with an *Aif1* promoter driving Cas9 expression. Knockdown constructs specifically affecting astrocytes were designed to target mouse *Gpr63* (Cat. No. CS-MCP002166-CG12-1-02-10) or mouse *Gpr153* (Cat. No. CS-MCP002167-CG12-1-02-10) with a *Gfap* promoter driving Cas9 expression. As a negative control (NC), a universal vector containing all the same elements as the above constructs and a nontargeting sgRNA was used. All constructs arrived in the form of transformed bacterial stabs.

Bacterial stocks prepared by streaking the initial stabs on LB agar/ampicillin (50µg/mL, 37°C) plates, culturing single colonies in LB broth with ampicillin (37°C, shaking), and preparing frozen stocks in 1:1 broth:glycerol solution, stored at -80°C. Bacteria stocks were recovered from frozen stocks by scraping the top of the frozen stock and incubating in 200 µL of S.O.C. Medium (Fisher Scientific, Cat. No. 15-544-034) for at least 5 hours but no more than 24 hours in an incubated shaker at 37°C. S.O.C. Medium containing propagated bacteria was plated on sterilized LB Agar (Fisher Scientific, Cat. No. BP9724-500) containing 50µg/mL ampicillin (Fisher Scientific, Cat. No. BP1760-25) and incubated overnight at 37 °C. Single colonies of plated bacteria were isolated and added to individual flasks containing 200 mL of sterilized LB Broth (Fisher Scientific, Cat. No. BP1426-500) with 50 µg/mL ampicillin and incubated overnight in an incubated shaker at 37°C. DNA plasmids containing CRISPR constructs were then isolated using EndoFree® Plasmid Maxi Kits (Qiagen, Cat. No. 12362) following the manufacturer's protocol. All constructs were administered at a dose of 1 µg DNA in a 5 µL volume (0.2 µg/µL) with GenJet *In Vivo* Transfection Reagent (SignaGen Laboratories, Cat. No. SL100500) according to the manufacturer's protocol. In brief, 20 µg of DNA (volume dependent on concentration of CRISPR construct DNA stock) was combined with 30 µL of 10% glucose in water and diluted to a total volume of 50 µL with nuclease-free water (Fisher Scientific, Cat. No. PR-P1193). Separately, 30 µL of GenJet *In Vivo* Transfection Reagent (1:1.5 ratio of DNA in µg to transfection reagent in µL) was combined with 20 µL of 10% glucose in water. The final concentration of the combined solution must be 5% glucose. Both solutions were combined, vortexed, and incubated for 10 minutes at room temperature then immediately administered to CD-1 mice. Constructs were delivered via the intrathecal (IT) route twice daily with at least 2 hours between injections for three consecutive

days resulting in a total 6 µg dose. All behavioral experiments or tissue collections were conducted on day 10 as in **Chapter 2**.

### **Behavioral Experiments**

Prior to behavioral experiments, animals were acclimated to the experimental setting (i.e., testing room and/or apparatus) for 30-60 minutes before any procedure. Mice were randomly block-assigned to treatment group by cage. Behavioral testing took place at the approximate same time of day between experiments while minimizing environmental factors (e.g., noise, personnel, and scents). Testing apparatuses were cleaned after each use. The experimenter was blinded to treatment groups for all behavioral experiments through coded animal cage labels generated by another laboratory member; decoding/unblinding occurred only after data collection was complete.

### **Chemotherapy-Induced Peripheral Neuropathy and Mechanical Allodynia**

The mice were housed in a homemade apparatus with Plexiglas walls and ceiling and a wire mesh floor (3"W x 4"L x 3"H with 0.25" wire mesh). Baseline mechanical thresholds were measured manually using calibrated von Frey filaments (Ugo Basile, Varese, Italy) to stimulate the left hind paw of each mouse. Mechanical thresholds were calculated using the up-down method with four measurements recorded following the first response per mouse on the first day of the experimental timeline before any drug treatment [17; 177].

For CRISPR experiments, mice were returned to the von Frey apparatus after the final injection on Day 3 and allowed to acclimate for 30-60 minutes. Post-CRISPR mechanical threshold measurements were recorded. Mice were then administered 2 mg/kg paclitaxel in a 1:1:4

mixture of Kolliphor EL, absolute ethanol, and USP Saline via the intraperitoneal (IP) route on Days 3, 5, 7, and 9 to induce CIPN. On Day 10, the development of CIPN was confirmed by observing a severe reduction (final threshold  $<0.5$  g) in the mechanical thresholds of each mouse compared to pre-CIPN baseline measurements. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were measured over a three-hour time course.

### **Paw Incision Surgery, Mechanical Allodynia, and Recovery**

Baseline mechanical threshold was measured for all mice using von Frey filaments as described above. Following baseline measurements, the mice were injected with CRISPR constructs as described above. On Day 9, post-CRISPR mechanical threshold measurements were taken. Immediately following the post-treatment mechanical threshold measurements, plantar paw incision surgery was performed on the left hind paw of each mouse as described in [178]. In brief, anesthesia of each mouse was induced with 3-5% isoflurane in room air and reduced to 1-3% for maintenance for the duration of the surgery. The left hind paw was sterilized by three repetitions of alternating iodine and 70% ethanol in water scrubs with a cotton swab. A 5mm incision was made through the skin and fascia using a No. 11 scalpel beginning just outside of the foot pads of the hind paw and extending towards the heel to expose the flexor digitorum brevis muscle. Fine tip curved forceps were inserted under the muscle and used to elevate the muscle out of the surgical wound. A separate No.11 scalpel was used to create incisions along the longitudinal axis of the muscle belly. The muscle was replaced and the wound was sutured using 5-0 vicryl sutures. Mice were administered 0.3 mg of gentamicin in USP Saline and 1mL of USP Saline SC and returned to their enclosure for post-operative monitoring. The following day (Day 10), mechanical

allodynia was evaluated by a severe reduction (final threshold < 0.5 g) in mechanical thresholds of each mouse compared to pre-surgery baseline measurements. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were recorded over a three-hour time course. Single time point measurements of recovery from surgery-induced mechanical allodynia in the absence of any drug treatment were then recorded on Days 11 and 12. Mice that did not develop mechanical allodynia post-surgery were excluded.

### **RNAScope™ *In Situ* Hybridization for Cell Type Colocalization**

#### *Tissue Collection and Preparation*

In separate cohorts from those used in CRISPR behavioral experiments, CRISPR DNA was administered in identical fashion to the behavioral experiments described above but naïve to the induction of any pathological pain state or opioid drug administration. On Day 10 following the beginning of CRISPR delivery, mice were perfused with chilled phosphate-buffered saline (PBS) for 5 minutes followed by chilled 4% paraformaldehyde (PFA) in PBS. Immediately after perfusion with PFA, the spinal column was removed from the mouse, and the spinal cord was ejected by inserting a syringe containing chilled PBS with a 30G needle into the caudal end of the spinal column and applying pressure. Spinal cords were post-fixed in 4% PFA overnight at 4°C. Spinal cords were then dehydrated in 15% sucrose in PBS overnight at 4°C followed by 30% sucrose in PBS overnight at 4°C. After dehydration, spinal cords were flash frozen in optimal cutting temperature (OCT) compound (Fisher Scientific, Cat. No. 23730571)) using liquid nitrogen. Tissue was sectioned using an Eprelia HM 525 cryostat at a thickness of 15-20 µm and

dry-mounted directly onto SuperFrost Plus™ slides (Fisher Scientific, Cat. No. 1255015). Slides with mounted tissue were left to air dry for at least 2 hours up to overnight at -20°C.

#### *RNAScope™ In-Situ Hybridization*

Prepared slides were allowed to equilibrate to room temperature and then washed with PBS until the excess OCT compound remaining from the mounting process was removed. Slides were baked at 60°C for 30 minutes in a VWR Mini-Incubator (Cat. No. 97025-630) then post-fixed in 4% PFA for 15 minutes at 4°C. Slides then underwent a dehydration series of 50% ethanol, 70% ethanol, and 100% ethanol twice for 5 minutes each at room temperature and then left to air dry. During the dehydration series, 1X Target Retrieval Buffer was prepared by diluting RNAScope™ 10X Target Retrieval Buffer (ACDBio, Cat. No. 322001, Lot 201955) with deionized water in a beaker. The beaker containing 1X Target Retrieval Buffer and a separate beaker containing deionized water were then placed in a Hamilton Beach Digital Steamer and left to warm. After drying, tissue sections were treated with RNAScope™ H<sub>2</sub>O<sub>2</sub> provided in the RNAScope™ H<sub>2</sub>O<sub>2</sub> and Protease Reagents Kit (ACDBio, Cat. No. 322381, Lot 2023923) at room temperature for 15 minutes and washed in deionized water for 2 minutes. The temperature of the 1X Target Retrieval Buffer was measured to ensure it was within 98-102°C. Slides were then acclimated to the high temperature in the beaker containing deionized water for 10 seconds before being transferred to the Target Retrieval Buffer and incubated for 15 minutes. Slides were washed in deionized water at room temperature for 10 seconds followed by incubation in 100% ethanol for 3 minutes and then left to air dry. After drying, a hydrophobic barrier was drawn around the tissue sections using

a Liquid Blocker Super Pap Pen (Electron Microscopy Sciences, Cat. No. 71310) and left to dry overnight at room temperature.

The following day, the incubator was set to 40°C and humidified by lining the bottom with dampened paper towels; paper towels were regularly dampened over the course of tissue treatment to maintain humidity in the incubator. Tissue sections were incubated with Protease III provided with the RNAScope™ H<sub>2</sub>O<sub>2</sub> and Protease Reagents Kit for 30 minutes at 40°C and washed in PBS for 2 minutes. Meanwhile, RNAScope™ 50X Wash Buffer (ACDBio, Cat. No. 320058, Lot 2018155) and RNAScope™ probes for targets of interest were also incubated at 40 °C for 15 minutes. Probes used for these experiments were designed by ACDBio to target mouse *Aif1* (Cat. No. 319141, Lot 23362B) and mouse *Gfap* (Cat. No. 313211, Lot 24009A) provided at 1X concentrations or mouse *Gpr63* (Mm-Gpr63-C2, Cat. No. 319311-C2) and mouse *Gpr153* (Mm-Gpr153-C2, Cat. No. 318101-C2) provided at 50X concentrations. After incubation, 50X probes and wash buffer were diluted to 1X using the 1X *Aif1* or *Gfap* probes and deionized water, respectively. Following protease treatment of tissue sections, slides were washed with PBS for 2 minutes. Target probes were then hybridized with the tissue sections by adding the prepared 1X RNAScope™ probe mixture and incubating at 40°C for 2 hours. After probe hybridization, slides were washed with 1X Wash Buffer for 2 minutes twice using fresh wash buffer each time. The following signal amplification workflow was performed using reagents from the RNAScope™ Multiplex Fluorescent Detection Kit (ACDBio, Cat. No. 323110, Lot 2023822) unless specified otherwise. Signal amplification took place by treating tissue sections with AMP1, AMP2, and AMP3 solutions for 30, 30, and 15 minutes, respectively, at 40°C with two 2-minute washes using fresh wash buffer in between each amplification step. Slides were then treated with HRP-C1 for

15 minutes at 40°C followed by two 2-minute washes using fresh wash buffer. The fluorescent signal was developed by treating tissue sections with a 1:250 dilution of Opal 520 (Akoya Biosciences, Cat. No. FP1497001KT, Lot 240627021) in RNAScope™ LS Multiplex TSA Buffer (ACDBio, Cat. No. 322810, 2019101) for 30 minutes at 40°C then washed with fresh wash buffer for 2 minutes twice. Tissue sections were then treated with HRP Blocker for 15 minutes and washed for 2 minutes twice with fresh wash buffer. Slides were then treated with HRP-C2 for 15 minutes at 40°C followed by two 2-minute washes using fresh wash buffer. The fluorescent signal was developed by treating tissue sections with a 1:250 dilution of Opal 620 (Akoya Biosciences, Cat. No. FP1495001KT, Lot 240619012) in RNAScope™ LS Multiplex TSA Buffer for 30 minutes at 40°C then washed with fresh wash buffer for 2 minutes twice. Tissue sections were then treated with HRP Blocker for 15 minutes and washed for 2 minutes twice with fresh wash buffer. Finally, tissue sections were stained with DAPI for 30 seconds and then immediately mounted using Fluoromount-G™ (Invitrogen, Cat. No. 00-4958-02, Lot E142722) with a No. 1 24X50 mm coverslip (Fisher Scientific, Cat. No. 12541042, Lot 23349) and sealed with clear nail polish (Ted Pella, Inc., Cat. No. 114-7). Fully prepared slides were stored in the dark at 4°C until ready for imaging.

### *Image Acquisition and Analysis*

RNAScope™ slides were imaged on an Olympus FV1200 laser scanning confocal microscope in the University of Arizona Department of Pharmacology. Images of the spinal dorsal horn were acquired using a 20x objective with filter cube configurations to allow imaging of DAPI, FITC (Opal 520), and Texas Red (Opal 620) emission spectra. Confocal settings were constrained

to the following parameters for all channels: HV  $\leq$  750, Gain  $\leq$  5, Offset  $\leq$  50. Image analysis was performed using QuPath software [179]. Regions of interest (ROIs) were drawn around the superficial lamina of the spinal dorsal horn and cell segmentation was performed by the software using the DAPI channel to identify individual nuclei. Cell boundaries were automatically drawn by the software using a set cell diameter of 10  $\mu$ m. QuPath software was then trained to count cells that expressed the GPR target of interest based on signal intensity within the cell boundaries. Cell counts were normalized by recording them as a proportion of the total cell count of the image. A minimum of three technical replicates were obtained for each biological replicate.

## **Immunohistochemistry for Microglia Morphology Analysis**

### *Tissue Collection and Preparation*

In separate cohorts from the CRISPR behavioral experiments described above, mice were perfused on Day 10 following CRISPR treatment with chilled phosphate-buffered saline (PBS) for 5 minutes followed by chilled 4% paraformaldehyde (PFA) in PBS. Immediately after perfusion with PFA, the spinal column was removed from the mouse, and the spinal cord was ejected by inserting a syringe containing chilled PBS with a 30G needle into the caudal end of the spinal column and applying pressure. Spinal cords were post-fixed in 4% PFA overnight at 4°C. Spinal cords were then dehydrated in 15% sucrose in PBS overnight at 4°C followed by 30% sucrose in PBS overnight at 4°C. After dehydration, spinal cords were flash frozen in optimal cutting temperature (OCT) compound (Fisher Scientific, Cat. No. 23730571)) using liquid nitrogen. Tissue was sectioned using an Eprelia HM 525 cryostat at a thickness of 50  $\mu$ m. Spinal cord

sections were rehydrated in PBS in preparation for free-float staining. Samples were incubated in an endogenous peroxidase blocking buffer consisting of 60% methanol and 0.3% H<sub>2</sub>O<sub>2</sub> in PBS at room temperature for 30 minutes and then washed with PBS + 0.1% Tween 20 (Sigma-Aldrich, Cat. No. 9005-64-5) (PBST). They were then incubated in a blocking buffer consisting of 10% donkey serum (Fisher Scientific, Cat. No. 50-588-37) and 0.3% Triton X-100 (Sigma Aldrich, Cat. No. 9002-93-1) in PBS at room temperature for 1 hour. Samples were then incubated with 1:100 primary Iba1 antibody (Abcam, Cat. No. AB 5076, Lot 1061058-2) in 1.5% donkey serum and 1% BSA (Fisher Scientific, Cat. No. BP1605-100) in PBST at 4°C overnight. Samples were then washed with PBST and incubated with a 1:400 secondary donkey anti-goat immunoglobulin G (IgG) Alexa Fluor 647 Plus antibody (Invitrogen, Cat. No. A32849, Lot ZK400520) in 1.5% donkey serum and 1% BSA in PBST at room temperature for 1 hour. The secondary antibody for Iba1 was Alexa Fluor donkey anti-goat IgG 647 was used at 1:400 and was added during the secondary incubation mentioned above. Spinal cord sections were stained with 300nM DAPI (Fisher Scientific, Cat. No. 57-481-0) for 30 seconds then washed twice with PBS for 5 minutes each and once with Nanopure water for 5 minutes. Sections were then mounted onto slides with Fluoromount-G mounting medium.

### *Image Acquisition and Analysis*

Sections were imaged using a 60x objective with 2.5-3x digital zoom using an Olympus FV4000 laser-scanning confocal microscope to obtain images of individual microglia. Three-dimensional images of microglia were acquired by collecting z-stacks spanning 30-40 µm with a 0.5 µm step size. Images were analyzed using Imaris 10 software. Soma were identified visually

and ROIs identifying soma for the software were generated manually. Microglia arborization was mapped using the Imaris software automated filament detection algorithm trained by the experimenter. Data collected to assess microglia complexity included segment branch depth, total sum of filament length, and soma volume.

### **RT-qPCR Analysis of oGPCR Expression in CIPN**

#### *Tissue Collection*

Mice received 2 mg/kg paclitaxel IP in identical fashion to the CIPN behavioral experiments described above. Whole spinal cord tissue for biochemical analysis was collected on Day 8 following the first IP injection. Mice were anesthetized using 5% isoflurane in room air and euthanized by manual cervical dislocation. The spinal column was removed from the mouse and the spinal cord was ejected by inserting a syringe containing chilled PBS with a 30G needle into the caudal end of the spinal column and applying pressure. Spinal cords were then immediately flash frozen using liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until the experimenter was prepared to perform the RNA isolation.

#### *RNA Isolation*

Spinal cord tissue samples were thawed on ice then homogenized manually with 1 mL of TRIzol reagent (Invitrogen, Cat. No. 15596018, Lot 563710) in a Dounce homogenizer. Tissue homogenates were then transferred to 1.5 mL Eppendorf tubes and incubated on ice for at least 5 minutes or overnight at  $4^{\circ}\text{C}$ . Tissue homogenates were then combined with 200  $\mu\text{L}$  of chloroform, mixed by shaking, and then incubated on ice for 3 minutes. Samples were centrifuged for 15

minutes at 12,000g at 4°C. The upper aqueous layer was carefully transferred to a separate 1.5 mL Eppendorf tube using a micropipette and the remaining layers were discarded. The aqueous layer was then combined with 500 µL of isopropanol and incubated for 30 minutes at 4°C. This mixture was then centrifuged for 10 minutes at 12,000g at 4°C to yield a white RNA pellet. RNA pellets were isolated by carefully aspirating the supernatant and resuspended in 1mL of 75% ethanol in nuclease-free water. The resuspended pellet was then centrifuged for 5 minutes at 7,500g at 4 °C, the supernatant was carefully aspirated again, and the pellet was air dried for 10 minutes. After drying, the RNA pellet was dissolved in 50 µL of nuclease-free water and incubated in a heating block at 55-60°C for 15 minutes. RNA concentration and quality was assessed using a NanoDropND-1000 spectrophotometer (NanoDrop Technologies) and stored at -80°C until the experimenter was prepared for the reverse transcription reaction.

#### *Reverse Transcription Reaction for cDNA Generation*

RNA samples and the High Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Cat. No. 4387406, Lot 2945899) were thawed on ice. The reverse transcription reaction mixture for each sample was composed of the following: 10 µL of 2X RT Buffer Mix, 1 µL of 20X RT Enzyme Mix, 1 µg of RNA (volume calculated based on sample concentration), and nuclease-free water in a quantity sufficient to dilute the total volume to 20 µL. Reverse transcription was performed using a BioRad C1000 Touch Thermal Cycler using the following parameters: 37°C for 60 minutes, 95°C for 5 minutes, 4°C for at least 10 minutes. Following the final 4°C cooling step, samples

were removed from the thermal cycler and stored at 4°C overnight or -20°C long term until the experimenter was prepared for real-time quantitative PCR analysis.

### *Real-Time Quantitative PCR and Quantification*

Real-time quantitative PCR was performed using a 96-well plate with each well containing a mixture of the following components: 0.3 µL of the target forward primer, 0.3 µL of the target reverse primer, 1 µL of cDNA, 10.9 µL of nuclease-free water, and 12.5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Cat. No. A25742, Lot 3089500). Each reaction was run in triplicate on a BioRad C1000 Touch Thermal Cycler using the following parameters: 95 °C for 10 minutes followed by 45 cycles of i) 95°C for 15 seconds, ii) 60°C for 1 minute followed by a final step at 72°C for 10 minutes. Relative RNA abundance was calculated by  $1/2^{\Delta\text{cycDiff}}$  of the GPR target of interest compared to internal GAPDH control and normalized to the NC siRNA treated control group from the same plate. Custom forward and reverse primers for mouse *Gpr63* and *Gpr153* were created by Integrated DNA Technologies (sequence information below). GAPDH primers were also obtained from IDT; sequence information for these can be found in [180].

#### *Primers:*

Mouse *Gpr63* Forward, Reference #460670820: (5' – TGG CTA CTC TGG CTC TGC TA – 3')

Mouse *Gpr153* Reverse Reference #460670821: (5' – CGA TGT TCC CCA CAC ACG TA – 3')

Mouse *Gpr153* Forward Reference #460670822: (5' – GCA GCA TTA GCA GCT TTC TGA – 3')

Mouse *Gpr153* Reverse Reference #460670823: (5' – TCC ACC ATC CAT TCA CCC CT – 3')

### Statistical Analysis

All data were reported as the mean  $\pm$  SEM. RT-qPCR data was normalized to an internal GAPDH control and RNAScope<sup>TM</sup> cell counts were normalized to the total cell counts (as determined from DAPI stain) from their respective ROIs. Behavioral data were reported raw with no maximum possible effect or other normalization applied. Statistical comparisons between groups were performed by Student's t-test (unpaired, two-tailed) for RNAScope<sup>TM</sup> and RT-qPCR experiments or two-way analysis of variance (ANOVA) with Dunnett's post hoc test for behavioral experiments. In all cases, statistical significance was defined as  $p < 0.05$ . All graphing and statistical analyses were performed using GraphPad Prism 9 software (San Diego, CA).

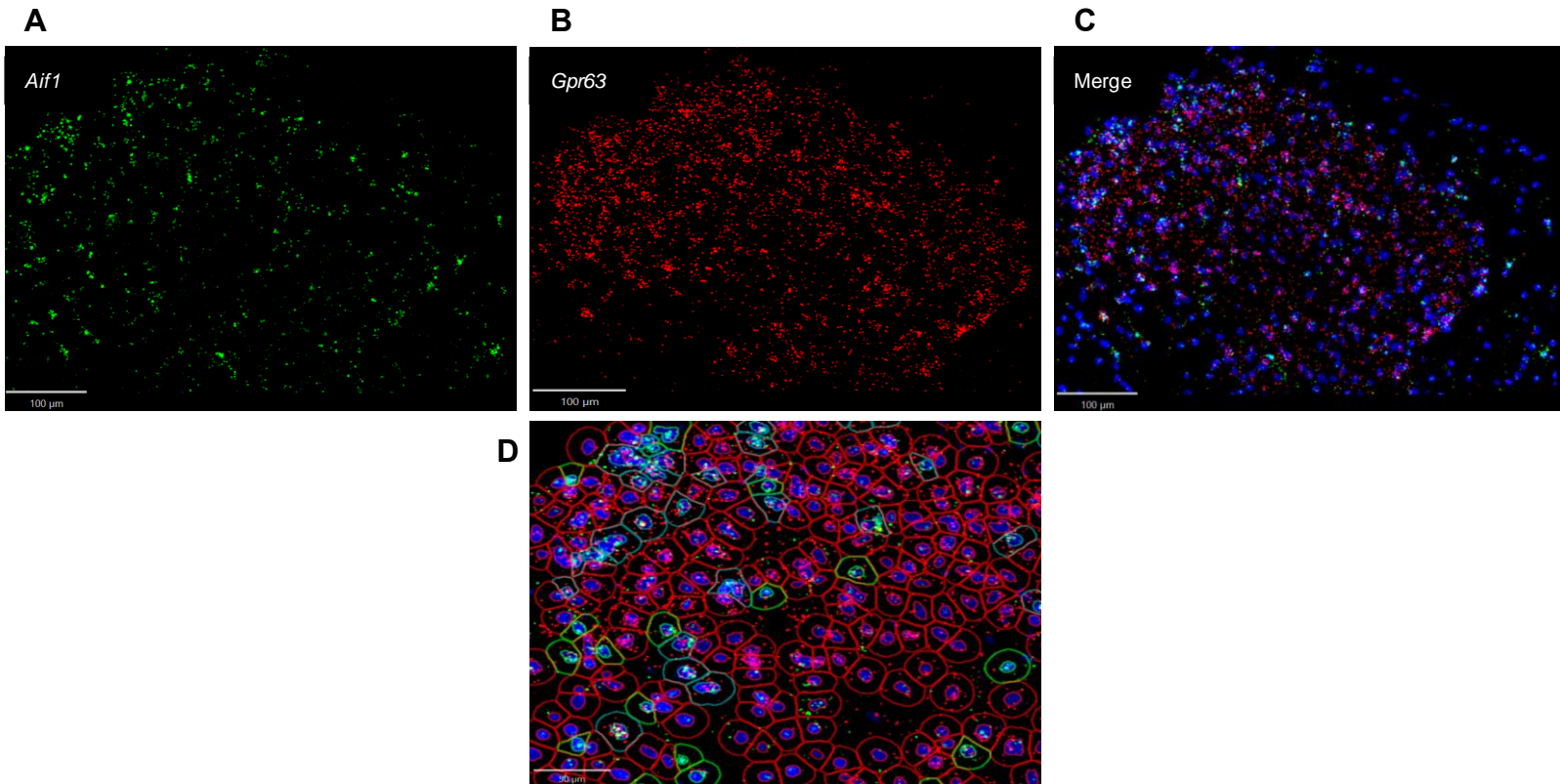
## 3.3: Results

### 3.3.1: GPR63 and GPR153 are Expressed in Both Microglia and Astrocytes

Currently, GPR63 and GPR153 expression has been confirmed in the human spinal cord by RNASeq analysis, but expression patterns are not yet well established in the CNS [143; 163]. Addressing this issue is further complicated by the lack of protein-targeting methods for these receptors to assess general localization of target expression such as immunohistochemistry (IHC).

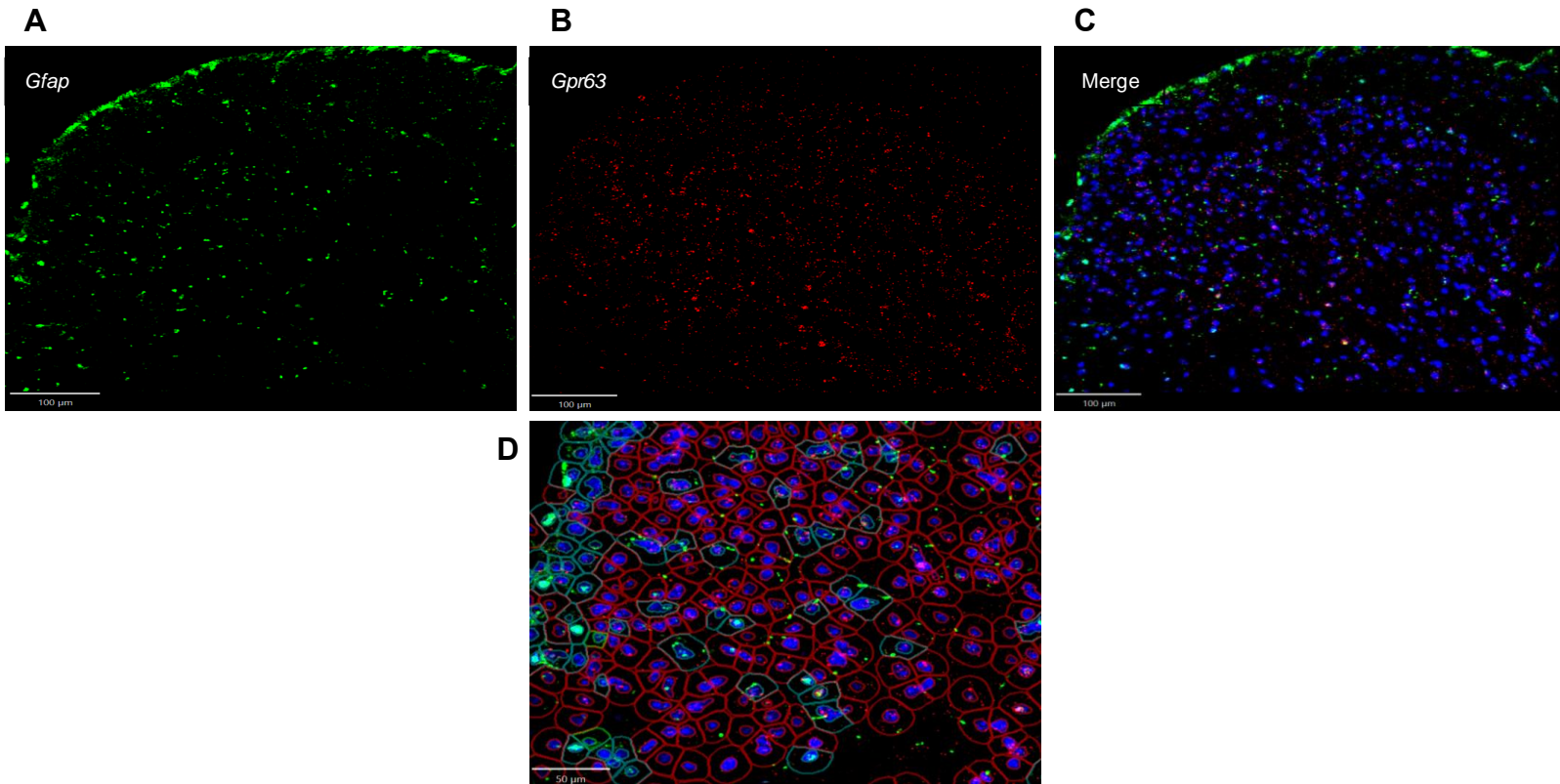
To remedy this critical gap in knowledge, we employed RNAScope™ *in situ* hybridization using probes for our oGPCR targets along with markers for microglia (*Aif1*) or astrocytes (*Gfap*). Probes targeting mouse *Gpr63* and *Gpr153* and glial cell marker mRNA transcripts were hybridized to the spinal cord tissue of male and female CD-1 mice naïve to any pain condition or drug treatment. Image analysis performed using QuPath software allowed us to segment individual cells and identify their phenotype based on levels of oGPCR and glial cell marker expression via presence of punctae located within the generated cell boundaries [179]. All combinations of RNAScope *in situ* hybridization were as follows: *Gpr63/Aif1*, *Gpr63/Gfap*, *Gpr153/Aif1*, and *Gpr153/Gfap*. Representative images of all combinations and examples of QuPath generated segmentation and colocalization are shown in **Figures 3.1 – 3.4**.

Expression of GPR63 and GPR153 was observed uniformly throughout the gray matter of the spinal dorsal horn suggesting that expression may not be specific to a single cell type and a complex biological mechanism may be underlying how these receptors interact with pathological pain and the endogenous opioid system. Nonetheless, receptor colocalization with glial cell markers was quantified. We observed that GPR63 was expressed in approximately 50% of all microglia and astrocytes in the spinal dorsal horn, providing a rather inconclusive result on whether the behavioral outcomes observed *in vivo* are mediated by one or the other (**Fig. 3.5 A and B**). By contrast, GPR153 expression was found in nearly all *Aif1*<sup>+</sup> cells and half of *Gfap*<sup>+</sup> cells, suggesting that GPR153 may play a role in modulating microglia function (**Fig. 3.5 C and D**).



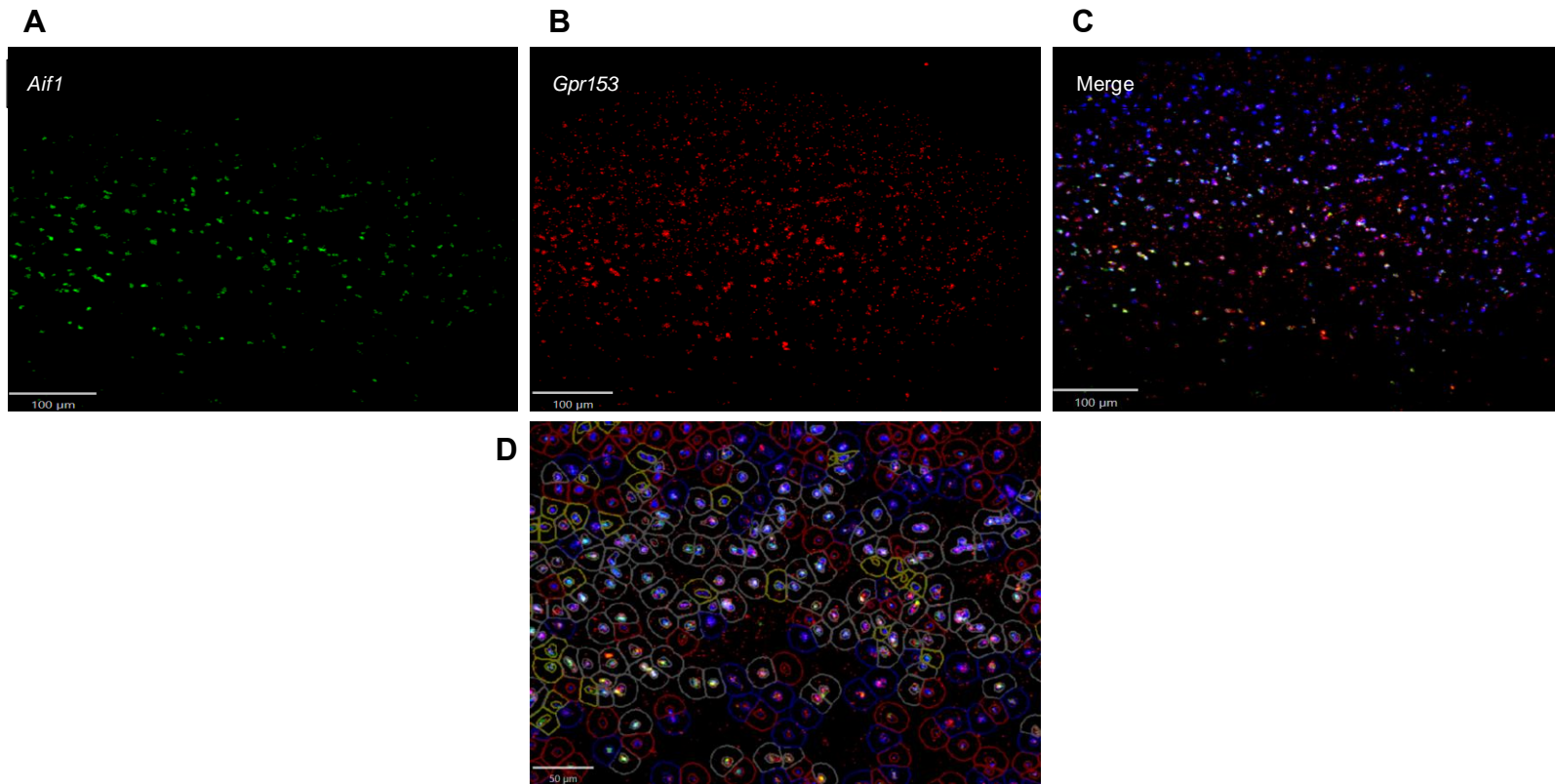
**Figure 3.1: RNAScope colocalization of *Gpr63* mRNA with the microglia marker *Aif1*.**

RNAScope *in-situ* hybridization of fixed spinal cord tissue from male and female CD-1 mice naïve to any pain condition or drug treatment was performed. Images were obtained using an Olympus Fluoview FV1200 laser-scanning confocal microscope using a 20x objective lens. All images taken were of the dorsal horn of the spinal cord. **(A)** *Aif1* (green) **(B)** *Gpr63* (red) **(C)** merged channels with DAPI counterstain (blue). Scale bars represent 100  $\mu\text{m}$ . **(D)** Images were processed using QuPath software. Cells were segmented by identifying nuclei using the DAPI counterstain and cell boundaries were automatically generated around each individual nucleus. Classifiers were programmed into the software to then group cells based on the presence of fluorescent signal corresponding to *Aif1*, *Gpr63*, both, or neither. Scale bar represents 50  $\mu\text{m}$ .



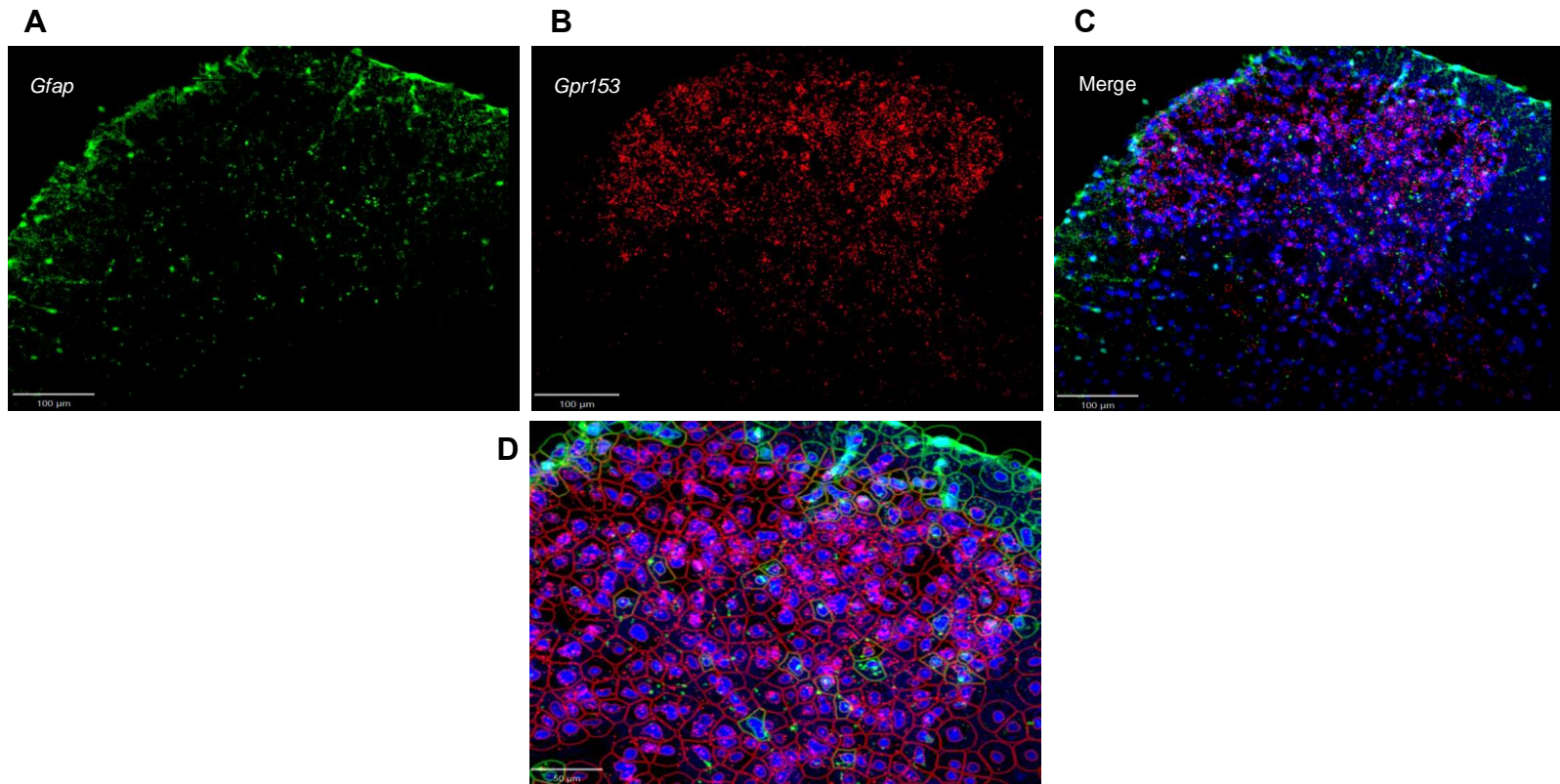
**Figure 3.2: RNAScope colocalization of *Gpr63* mRNA with the astrocyte marker *Gfap*.**

RNAScope *in-situ* hybridization of fixed spinal cord tissue from male and female CD-1 mice naïve to any pain condition or drug treatment was performed. Images were obtained using an Olympus Fluoview FV1200 laser-scanning confocal microscope using a 20x objective lens. All images taken were of the dorsal horn of the spinal cord. (A) *Gfap* (green) (B) *Gpr63* (red) (C) merged channels with DAPI counterstain (blue). Scale bars represent 100  $\mu\text{m}$ . (D) Images were processed using QuPath software. Cells were segmented by identifying nuclei using the DAPI counterstain and cell boundaries were automatically generated around each individual nucleus. Classifiers were programmed into the software to then group cells based on the presence of fluorescent signal corresponding to *Gfap*, *Gpr63*, both, or neither. Scale bar represents 50  $\mu\text{m}$ .



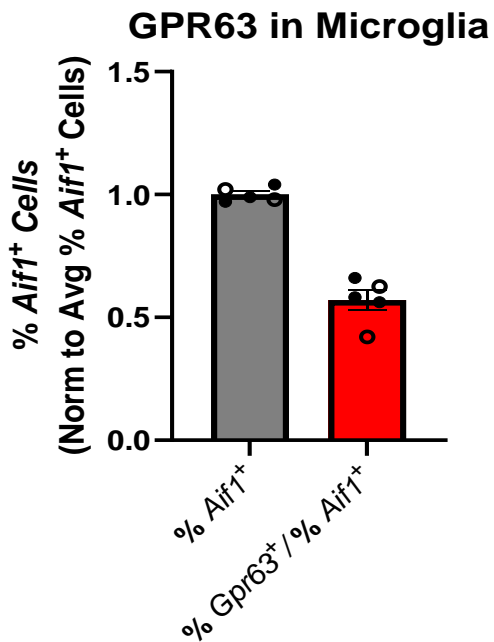
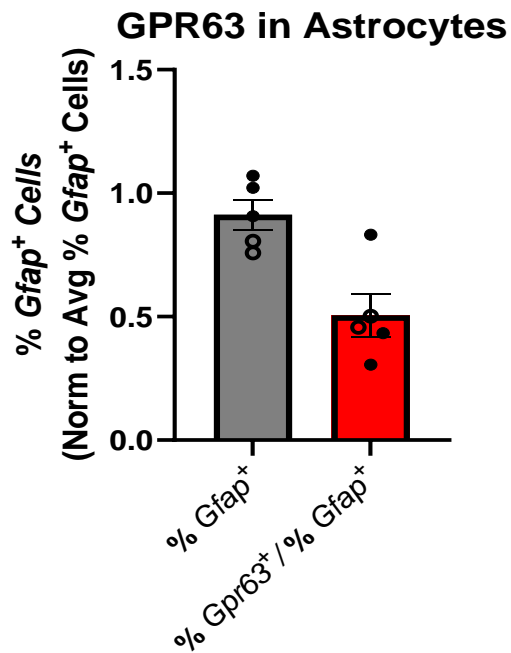
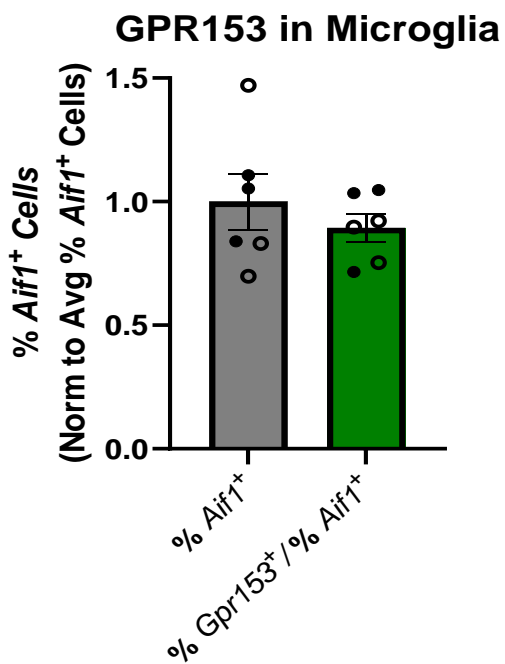
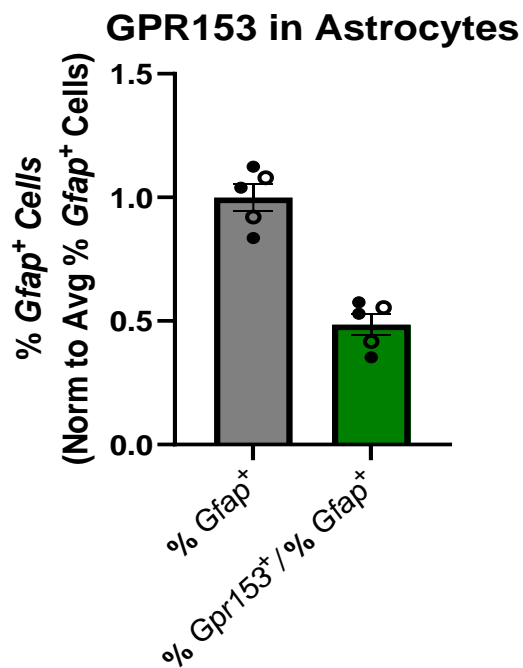
**Figure 3.3: RNAScope colocalization of *Gpr153* mRNA with the microglia marker *Aif1*.**

RNAScope *in-situ* hybridization of fixed spinal cord tissue from male and female CD-1 mice naïve to any pain condition or drug treatment was performed. Images were obtained using an Olympus Fluoview FV1200 laser-scanning confocal microscope using a 20x objective lens. All images taken were of the dorsal horn of the spinal cord. (A) *Aif1* (green) (B) *Gpr153* (red) (C) merged channels with DAPI counterstain (blue). Scale bars represent 100 μm. (D) Images were processed using QuPath software. Cells were segmented by identifying nuclei using the DAPI counterstain and cell boundaries were automatically generated around each individual nucleus. Classifiers were programmed into the software to then group cells based on the presence of fluorescent signal corresponding to *Aif1*, *Gpr153*, both, or neither. Scale bar represents 50 μm.



**Figure 3.4: RNAScope colocalization of *Gpr153* mRNA with the astrocyte marker *Gfap*.**

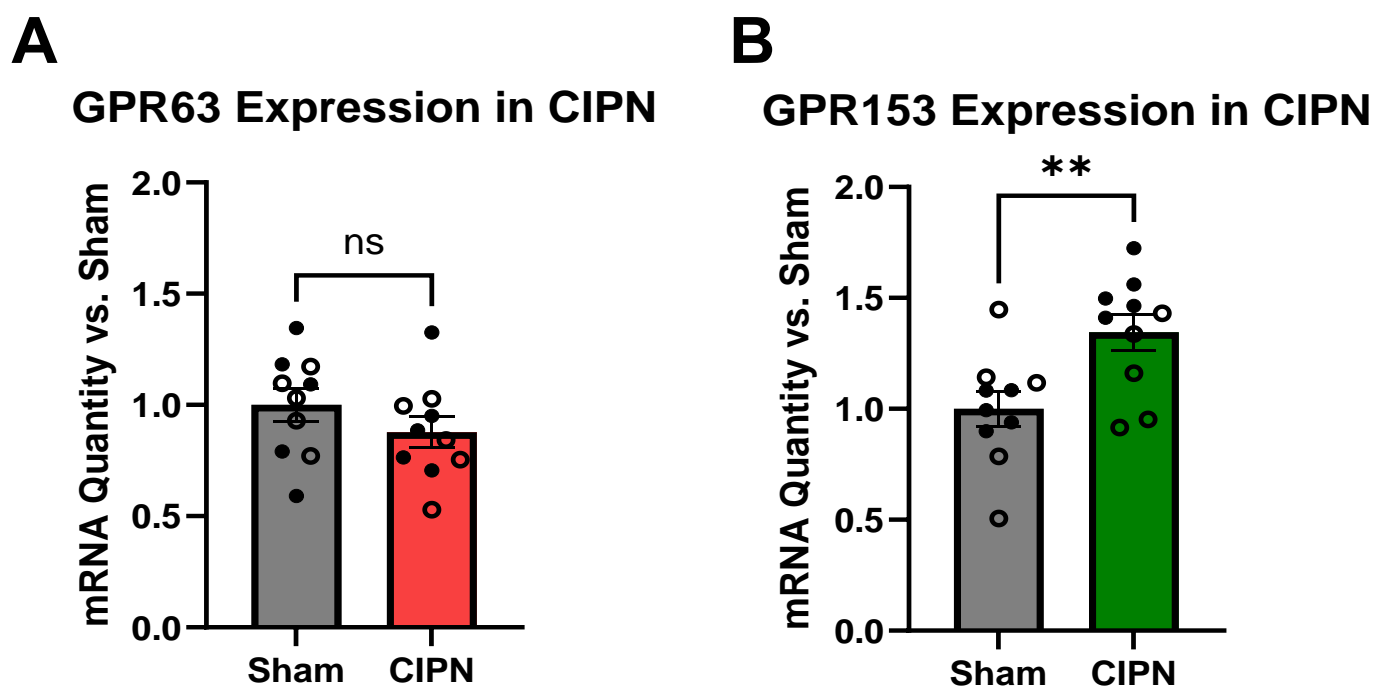
RNAScope *in-situ* hybridization of fixed spinal cord tissue from male and female CD-1 mice naïve to any pain condition or drug treatment was performed. Images were obtained using an Olympus Fluoview FV1200 laser-scanning confocal microscope using a 20x objective lens. All images taken were of the dorsal horn of the spinal cord. (A) *Gfap* (green) (B) *Gpr153* (red) (C) merged channels with DAPI counterstain (blue). Scale bars represent 100  $\mu\text{m}$ . (D) Images were processed using QuPath software. Cells were segmented by identifying nuclei using the DAPI counterstain and cell boundaries were automatically generated around each individual nucleus. Classifiers were programmed into the software to then group cells based on the presence of fluorescent signal corresponding to *Gfap*, *Gpr63*, both, or neither. Scale bar represents 50  $\mu\text{m}$ .

**A****B****C****D**

**Figure 3.5: Quantification of GPR63 and GPR153 colocalization with microglia and astrocytes by RNAScope *in-situ* hybridization.** Imaging data of RNAScope *in-situ* hybridization for colocalization of oGPCRs with glial cell markers was processed using QuPath software. Total cell counts were obtained by automated cell segmentation using the DAPI counterstain. Cells were then classified based on the presence of markers for glial cell types (i.e. *Aif1* or *Gfap*), oGPCR mRNA, both, or neither. All data points represent individual biological replicates (1 mouse) which are averaged values from three technical replicates each. Open symbols represent female mice and closed symbols represent male mice. **(A)** Approximately 50% of total spinal cord microglia express GPR63. **(B)** Approximately 50% of total spinal cord astrocytes express GPR63. **(C)** Nearly all spinal cord microglia express GPR153 with almost 100% equal cell counts of *Aif1* and *Gpr153/Aif1* double positive cells. **(D)** Approximately 50% of total spinal cord astrocytes express GPR153.

### 3.3.2: GPR153 but not GPR63 Expression is Increased in a Mouse Model of CIPN

It has been established that pathological pain states such as CIPN result in widespread changes in all cell types of the central and peripheral nervous system including organelle structure and function, cytokine release patterns, and intracellular signaling patterns amongst other crucial mediators of cell physiology [241-245]. In particular, changes in expression levels of numerous receptors have been observed in CIPN models including those that are tightly linked to glial cells such as TLR4 or various cytokine receptors [241; 246; 247]. Considering this, we investigated whether our CIPN model using paclitaxel would alter expression levels of GPR63 and GPR153 via RT-qPCR. We found that GPR63 expression did not change in the spinal cord using our model (**Fig. 3.6A**). We did, however, note that GPR153 expression increased by approximately 40% in the mouse spinal cord when undergoing the same treatment (**Fig. 3.6B**) which may indicate that altered signaling levels of this receptor are potentially involved in the pathology of paclitaxel-induced neuropathy, perhaps as an upregulated negative feedback loop on inflammation and pain.



**Figure 3.6: Expression of GPR153 but not GPR63 is increased in a mouse model of CIPN.**

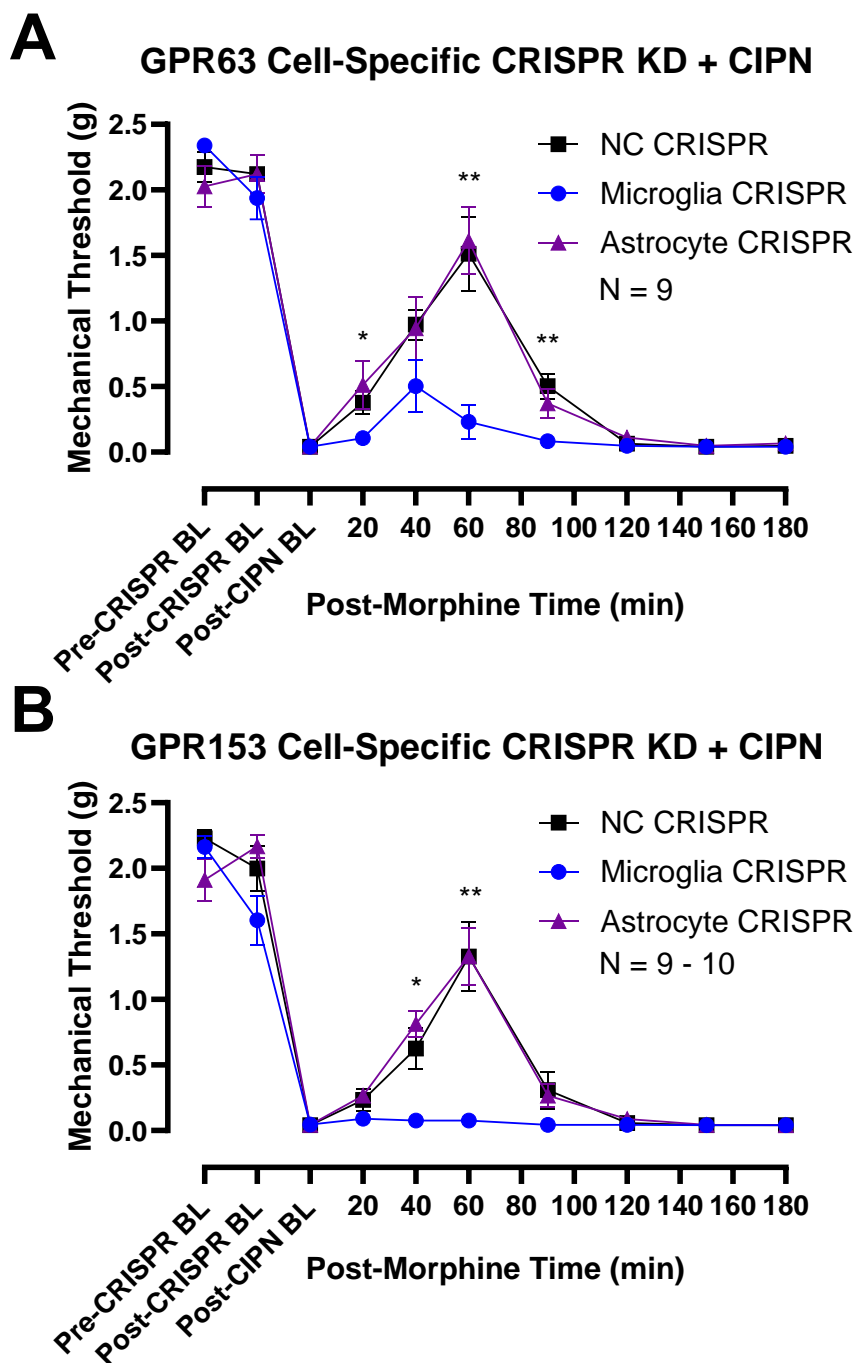
Male and female CD-1 mice had CIPN induced by administration of 2 mg/kg paclitaxel as described in the Methods. The development of CIPN was confirmed by assessing mechanical threshold using von Frey filaments before and after paclitaxel administration. All animals in this study developed mechanical allodynia. Whole spinal cord samples were extracted after CIPN induction and RT-qPCR was performed as described in the Methods. Open symbols represent female mice and closed symbols represent male mice. Data reported as mean  $\pm$  SEM. \*\* $p < 0.01$  vs. Sham treated group by unpaired 2-tailed Student's  $t$  test. **(A)** Induction of CIPN had no significant effect on the expression of GPR63 in the mouse spinal cord. **(B)** Induction of CIPN resulted in an approximately 40% increase in expression of GPR153 in the mouse spinal cord.

### 3.3.3: Microglia-Specific Knockdown of GPR63 and GPR153 Ablates Opioid-Induced Antinociception in a Mouse Model of CIPN Whereas Astrocyte-Specific Knockdown has no Effect

Thus far we have shown that GPR63 and GPR153 are expressed throughout the spinal dorsal horn and have relevant expression levels in both astrocytes and microglia with the strongest piece of evidence relating our targets to glial cells being that almost all microglia seem to express GPR153. This finding, however, does not provide us with any insights into how GPR63 and GPR153 may be modulating the activity of astrocytes and/or microglia in the pathological pain states we have interrogated them with. Lacking a ligand for either receptor complicates matters further as we are unable to develop hypotheses regarding the outcomes of their activity on glial cells without any context of their G-protein coupling or downstream signaling pathways. Nonetheless, we returned to genetically targeted and behavioral pharmacology approaches to attempt to gain further insights.

Similar to the work presented in **Section 2.3.2**, we performed CRISPR knockdowns of GPR63 and GPR153 and induced CIPN in CD-1 mice; the difference in this set of experiments being a cell-specific CRISPR approach. Previous CRISPR constructs used in this work contained a cytomegalovirus (CMV) promoter driving the expression of the Cas9 protein across all cell types to yield a global knockdown. The CMV promoter can be replaced with the promoter for other proteins allowing us to target Cas9 expression to cells with specific expression profiles; our lab has used this approach previously [15]. To target CRISPR knockdown to astrocytes and microglia, we replaced the CMV promoter with promoters for the *Gfap* and *Aif1* genes, respectively. We then sought to reevaluate the effect of target knockdown on opioid-induced antinociception when knockdown was only restricted to either astrocytes or microglia. Using this approach, we observed

that the knockdown of both GPR63 and GPR153 again ameliorated the antinociceptive effects of morphine when the knockdown only targeted microglia (**Fig. 3.7**). Astrocyte-specific knockdown of both targets interestingly had no effect and morphine demonstrated an analgesic effect comparable to that seen in the NC CRISPR-treated cohort. This finding now provides the first piece of evidence to potentially uncover the biological mechanism by which GPR63 and GPR153 knockdown interact with the pathology of CIPN and the effects of opioids in the context of this pain model.

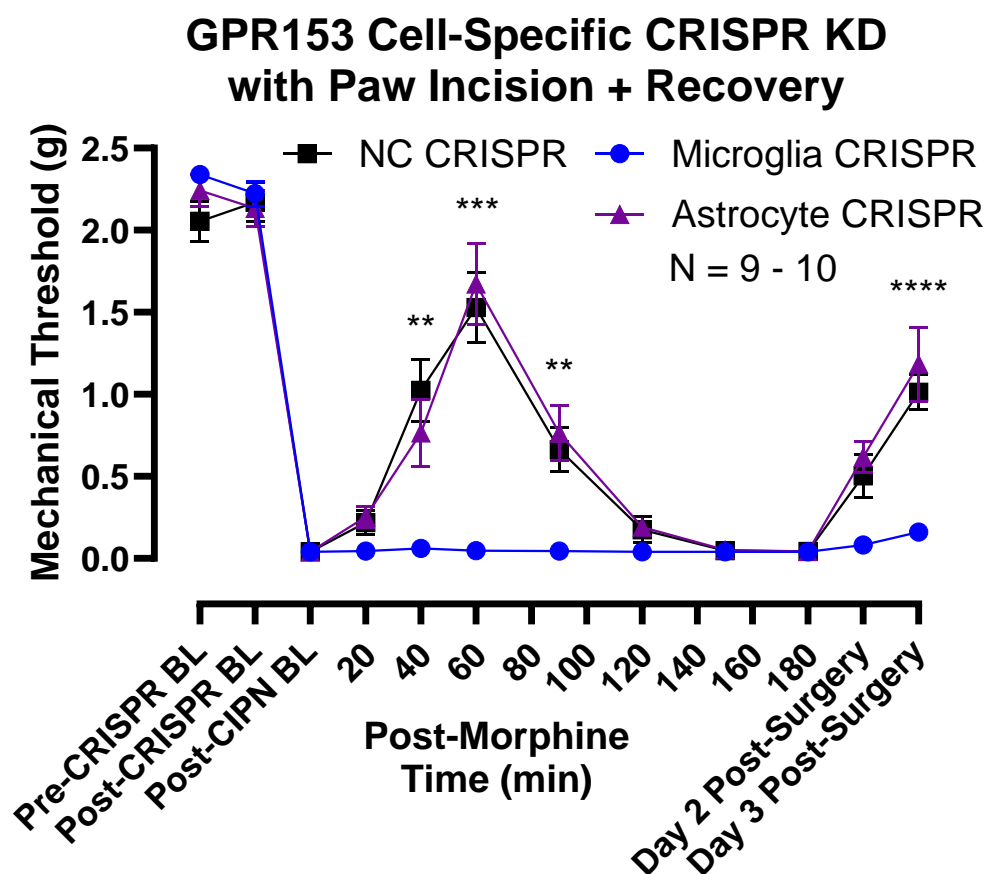


**Figure 3.7: Microglia-specific CRISPR knockdown of GPR63 and GPR153 ablates opioid-induced antinociception in a mouse model of CIPN whereas astrocyte-specific knockdown has no effect.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63 or (B) GPR153 specifically targeted to astrocytes or microglia alongside a separate cohort of mice that received a negative control (NC) CRISPR construct via IT injection as

described in the Methods. Pre-CRISPR mechanical threshold baseliens were measured using calibrated von Frey filaments immediately prior to the first IT injection, and post-CRISPR mechanical thresholds were measured after the final IT injection after animals were allowed to reacclimate to the von Frey testing apparatus. Immediately following the post-CRISPR baseline measurement, mice received 2 mg/kg paclitaxel IP in Days 3, 5, 7, and 9. On Day 10, the development of mechanical allodynia was confirmed by a dramatic decrease in mechanical threshold; no animals were excluded for failing to develop mechanical allodynia. Mice were treated with 3.2 mg/kg morphine SC and mechanical thresholds were evaluated over a three-hour time course. Data reported as mean  $\pm$  SEM. \*,\*\*p < 0.05, 0.01 vs. NC CRISPR group at the same time point by repeated measures two-way ANOVA with with Dunnett's post hoc test. CRISPR knockdown of **(A)** GPR63 and **(B)** GPR153 abolished the antinociceptive effects of morphine only when targeted to microglia but not astrocytes suggesting that the biological mechanism of this result is mediated through the activity of microglia in some capacity.

### 3.3.4: Microglia-Specific Knockdown of GPR153 Abolishes Opioid-Induced Antinociception and Impairs Recovery from Mechanical Allodynia in a Mouse Model of Post-Operative Pain but Astrocyte-Specific Knockdown has no Effect

Our behavioral screening efforts in the previous chapter also demonstrated that knockdown of GPR153 but not GPR63 interfered with opioid-induced antinociception in a mouse model of post-surgical pain. Additionally, the knockdown of GPR153 also impaired the normal recovery trajectory from mechanical allodynia which may implicate GPR153 dysfunction as a key mediator in the transition from normal nociceptive pain to a maladaptive persistent pain condition. To determine if this effect was driven by the activity of GPR153 in a specific glial cell type, we again performed cell-specific CRISPR knockdown targeting astrocytes and microglia. In line with our findings in **Section 3.3.3**, GPR153 knockdown targeted to astrocytes had no impact on the effects of morphine treatment to relieve post-surgical mechanical allodynia in mice. Microglia-specific knockdown of GPR153, on the other hand, prevented the manifestation of any pain relief from the administered dose of morphine (**Fig. 3.8**). Recovery from post-surgical mechanical allodynia was unaffected by the astrocyte-specific knockdown whereas hypersensitivity to mechanical stimuli persisted in mice that received the microglia-specific knockdown. With this, we have not only replicated the findings of our previous global knockdown using the paw incision model but also show evidence that the observed effects are also mediated by microglial function as in our CIPN results.



**Figure 3.8: Microglia-specific knockdown of GPR153 abolishes opioid-induced antinociception and impairs recovery from mechanical allodynia in a mouse model of post-operative pain but astrocyte-specific knockdown has no effect.** Male and female CD-1 mice received CRISPR knockdown of GPR153 specifically targeted to astrocytes or microglia alongside a separate cohort of mice that received a negative control (NC) CRISPR construct via IT injection as described in the Methods. Pre-CRISPR mechanical threshold baselines were measured using calibrated von Frey filaments immediately prior to the first IT injection, and post-CRISPR baseline measurements were taken on Day 9 immediately prior to the post-surgical pain model. The

following day (Day 10), mechanical allodynia caused by the surgical wound was confirmed via von Frey; no animals were excluded for failing to develop mechanical allodynia. Mice were then treated with 3.2 mg/kg morphine SC and mechanical thresholds were evaluated over a three-hour time course. Single time point measurements in the absence of morphine were then taken on Days 11 and 12 to measure recovery from post-surgical mechanical allodynia. CRISPR knockdown of GPR153 completely ablated the antinociceptive effects of morphine only when the knockdown was targeted to microglia but not astrocytes. GPR153 knockdown specific to microglia also impaired recovery from post-surgical mechanical allodynia. Data reported as mean  $\pm$  SEM. \*\*,\*\*\*,\*\*\*\*p < 0.01, 0.001, 0.0001 vs. NC CRISPR group at the same time point by repeated measures two-way ANOVA with Dunnett's post hoc test.

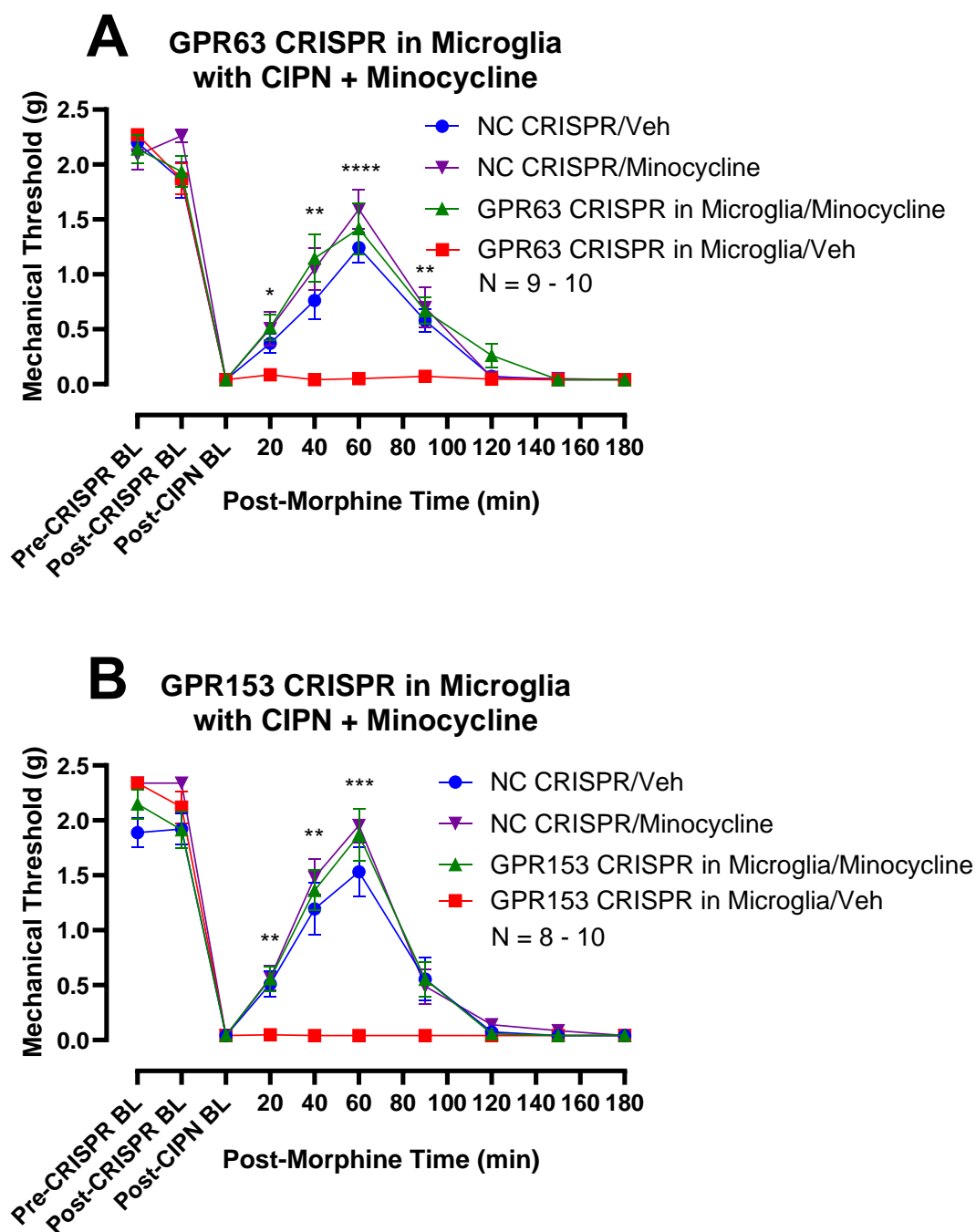
### 3.3.5: Inhibition of Microglia with Minocycline Reverses the Blockage of Opioid-Induced Antinociception Following Microglia-Specific Knockdown of GPR63 and GPR153 in CIPN

Through our cell-specific CRISPR knockdown paradigm, we have hypothesized that the biological mechanism that leads to GPR63 and GPR153 knockdown blocking opioid-induced antinociception in pathological pain states is at least in part mediated by the modulation of microglial function in the spinal cord. As mentioned in the introduction of this chapter, microglia play a crucial role in the development of persistent pain by their participation in neuroinflammatory cascades that sensitize neurons of the nociceptive circuit. Microgliosis is typically triggered in response to stimulatory signals that are indicative of injury in the local environment (e.g., ATP released from damaged cells and stimulating purinergic receptors on microglia) and/or the loss of inhibitory signals that healthy cells normally provide (e.g., cell-surface recognition proteins) to keep microglia inactive [248-251]. Microglial activation is tightly linked to the onset of pathological pain states [40; 203; 219; 220]. Taking the current view of the role of microglia in pathological pain states in conjunction with our observations that GPR63 and GPR153 knockdown prevents opioid-induced pain relief *in vivo*, we hypothesized that this outcome may result from increased microgliosis following target receptor knockdown.

To interrogate this hypothesis, we returned to our CRISPR knockdown paradigm with CIPN and introduced the microglial inhibitor minocycline. Minocycline was originally introduced as an antibiotic drug but has since been found to also exert anti-inflammatory effects including efficacy in curtailing neuroinflammation [252-254]. Specifically for microglia, minocycline has been shown to mitigate microgliosis, prevent microglial polarization, and reduce neuroinflammation and phagocytosis demonstrated by polarized microglia in CNS disease states

by targeting pathways necessary for antigen recognition and expression of pro-inflammatory mediators [255-257]. Minocycline has also been directly considered as a potential therapeutic for certain pathological pain states like CIPN in animal studies, but current clinical data demonstrating efficacy is inconclusive [258; 259].

Our CRISPR knockdown of GPR63 and GPR153 was once again targeted specifically to microglia and CIPN was induced in CD-1 mice. Minocycline was then delivered to the spinal cord via the IT route alongside separate cohorts of mice that received minocycline vehicle (USP Sterile Water) treatment. We observed that the knockdown of both GPR63 and GPR153 when targeted to microglia again prevented opioid-induced antinociception from presenting in mice (**Fig. 3.9**; red traces). Mice treated with NC CRISPR and either minocycline or vehicle both demonstrated normal analgesic responses to morphine as well (**Fig. 3.9**; blue and purple traces). Pleasantly, the mice that received knockdown of our targets in microglia and then received minocycline treatment also experienced pain relief comparable to the NC CRISPR groups (**Fig. 3.9**; green traces). We did not observe any synergistic or additive antinociception through the combination of minocycline and morphine in the NC CRISPR treated groups, however. This finding thus provides the first insight into the potential physiological impacts of GPR63 and GPR153 on microglia and supports our hypothesis that the loss of GPR63/153 leads to enhanced microglial activation.



**Figure 3.9: Inhibition of microglia using minocycline reverses the loss of opioid-induced antinociception caused by microglia-specific GPR63 and GPR153 CRISPR knockdown in a mouse model of CIPN.** Male and female CD-1 mice received CRISPR knockdown of a single target oGPCR (A) GPR63 or (B) GPR153 specifically targeted to microglia alongside a separate

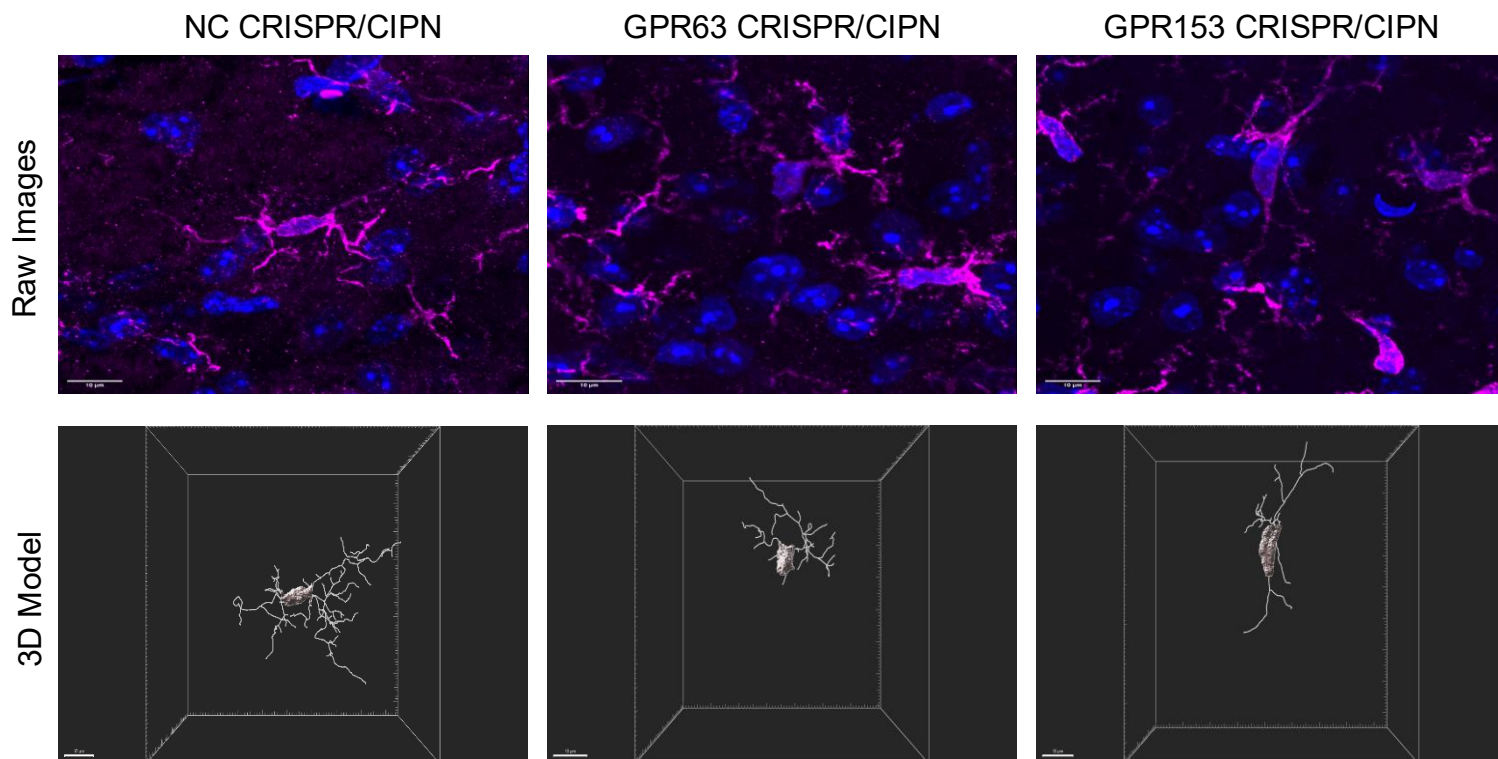
cohort of mice that received a negative control (NC) CRISPR construct via IT injection as described in the Methods. Pre-CRISPR mechanical threshold baseliens were measured using calibrated von Frey filaments immediately prior to the first IT injection, and post-CRISPR mechanical thresholds were measured after the final IT injection after animals were allowed to reacclimate to the von Frey testing apparatus. Immediately following the post-CRISPR baseline measurement, mice received 2 mg/kg paclitaxel IP on Days 3, 5, 7, and 9. On Day 10, the development of mechanical allodynia was confirmed by a dramatic decrease in mechanical threshold; no animals were excluded for failing to develop mechanical allodynia. Mice then received a 100 µg dose of minocycline IT. Thirty minutes after minocycline administration, mice were treated with 3.2 mg/kg morphine SC and mechanical thresholds were evaluated over a three-hour time course. Data reported as mean ± SEM. \*,\*\*,\*\*\*,\*\*\*\*p < 0.05, 0.01, 0.001, 0.0001 vs. NC CRISPR group at the same time point by repeated measures two-way ANOVA with Dunnett's post hoc test. Treatment of mice with CRISPR knockdown of (A) GPR63 or (B) GPR153 specific to microglia by minocycline reinstated the antinociceptive effect of morphine (green traces). Conversely, morphine showed no antinociceptive effect in mice that received knockdown of either target but did not receive minocycline (red traces).

### 3.3.6: GPR63 and GPR153 CRISPR Knockdown Alters Microglia Morphology in a Mouse Model of CIPN

Other hallmarks of microgliosis in CNS pathologies (e.g., persistent pain conditions) include distinct changes in morphology. In resting states, microglia can be characterized as having small cell bodies with numerous long and highly ramified projections that are used to monitor the extracellular environment [260]. Microglia respond to nerve injury and other conditions that lead to pathological pain by shifting to larger amoeboid cell bodies with shorter projections and becoming more numerous in quantity either through migration or proliferation [251; 260; 261]. Treatments targeting neuroinflammation in CNS disease states have also exhibited effects on microglia that promote healthy resting state phenotypes [261; 262]. Following our hypothesis that loss of GPR63 and GPR153 in microglia somehow worsens microgliosis in our CIPN model, we sought to investigate whether target knockdown impacts microglia morphology directly.

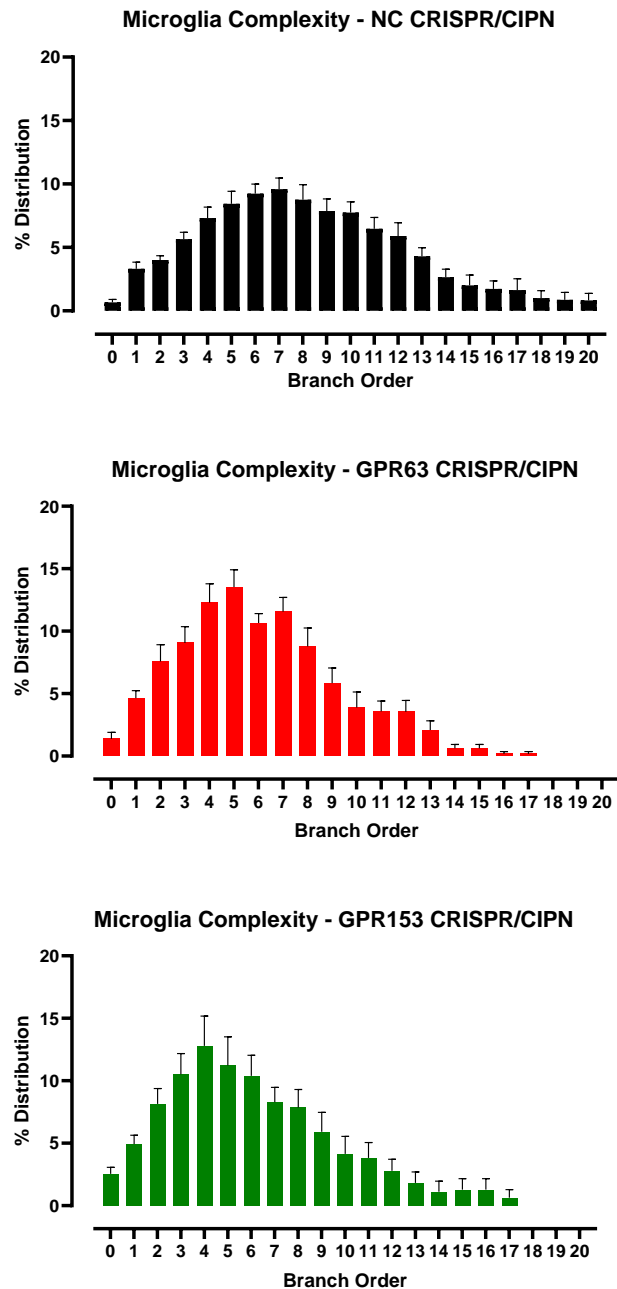
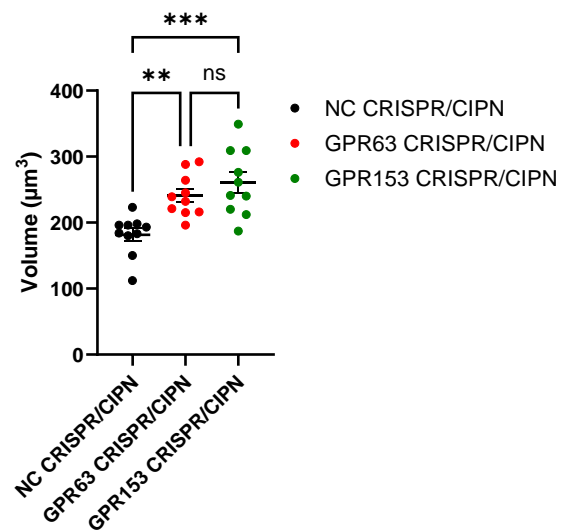
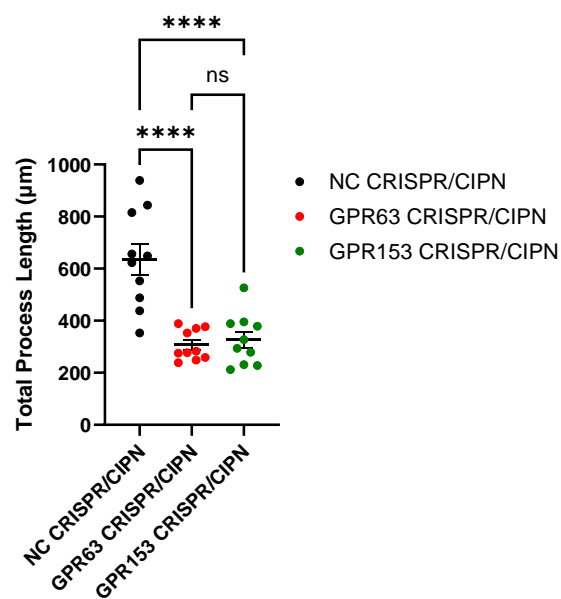
Microglia-specific knockdowns of GPR63 and GPR153 were performed and CIPN was induced in CD-1 mice as in all previous CIPN experiments. Immunohistochemical staining for microglia (Iba1) was performed and imaged by laser-scanning confocal microscopy to capture the 3D structure of microglia in the spinal dorsal horn for morphological analysis. Images were then analyzed and 3D models were generated using Imaris 10 software for quantification of morphological differences. Upon visual inspection, it was notable that microglia from the NC CRISPR/CIPN had more complex arborization whereas GPR63 and GPR153 CRISPR-treated microglia had larger soma, shorter and less complex processes, and higher density within the same field of view (**Fig. 3.9**). Quantification of arbor complexity was assessed by measuring the distribution of branch order for all ramifications extending from the soma; branch order for a given

branch within the microglial arbor was defined as the number of ramification points preceding that branch. Branch order as a metric for microglial complexity was found to be statistically different by two-way ANOVA ( $F_{(20, 561)} = 43.32$ ,  $p < 0.0001$ ) with both GPR63 and GPR153 CRISPR-treated microglia having more ramifications at lower branch orders compared to the negative control (**Fig. 3.10A**). Soma volume was significantly increased in target knockdown groups indicative of a more amoeboid soma shape consistent with stronger microgliosis (**Fig. 3.10B**). The total process length of GPR63 and GPR153-treated microglia was also significantly decreased which is congruent with the finding of decreased arborization complexity (**Fig. 3.10C**). In total, we have collected evidence that the reduction of GPR63 and GPR153 expression in spinal cord microglia using a CIPN model results in a stronger neuroinflammatory microglial phenotype. Further, since we can rescue the impacts on antinociception after knockdown with minocycline treatment, our findings suggest the GPR63/153 impacts on microglial function regulate pathological pain relief.



**Figure 3.10: CRISPR knockdown of GPR63 and GPR153 in a mouse model of CIPN alters microglia morphology.** Female CD-1 mice received CRISPR knockdown of a single target oGPCR GPR63 or GPR153 specifically targeted to microglia alongside a separate cohort of mice that received an NC CRISPR construct via IT injection as described in the Methods. Immediately following the final CRISPR administration, CIPN was induced by administering 2 mg/kg paclitaxel IP on Days 3, 5, 7, and 9 and mice were fixed via transcardial perfusion on Day 10. Immunohistochemistry was performed on fixed spinal cord tissue staining for Iba1 (magenta) with a DAPI counterstain (blue). Individual microglia were identified and imaged using an Olympus FV4000 laser-scanning confocal microscope and a 60x oil objective with 2.5-3x digital zoom. 3D images were generated by Z-stacks spanning a 30-45  $\mu\text{m}$  range. Microglia were approximately centered in the field of view when imaged. Imaris 10 software was then used to process microglia Z-stack images and generate 3D models of the soma and arborization patterns. Raw images of

maximum intensity Z-projections are shown in the top row for NC (left), GPR63 (middle), and GPR153 (right) CRISPR-treated groups. The corresponding 3D models generated in Imaris 10 are shown in the bottom row. Qualitatively, it is notable that GPR63 and GPR153 CRISPR treatments resulted in soma with larger volumes and less complex arborization patterns compared to their NC CRISPR-treated counterparts. This suggests that CRISPR knockdown of target oGPCRs results in stronger microgliosis indicative of a worse pathological outcome.

**A****B****C**

**Figure 3.11: Quantification of alterations to microglia morphology following CRISPR knockdown of GPR63 and GPR153 in a mouse model of CIPN.** Female CD-1 mice received CRISPR knockdown of a single target oGPCR GPR63 or GPR153 specifically targeted to

microglia alongside a separate cohort of mice that received an NC CRISPR construct via IT injection as described in the Methods. Immediately following the final CRISPR administration, CIPN was induced by administering 2 mg/kg paclitaxel IP on Days 3, 5, 7, and 9 and mice were fixed via transcardial perfusion on Day 10. Immunohistochemistry was performed on fixed spinal cord tissue staining for Iba1 (magenta) with a DAPI counterstain (blue). Individual microglia were identified and imaged using an Olympus FV4000 laser-scanning confocal microscope and a 60x oil objective with 2.5-3x digital zoom. 3D images were generated by Z-stacks spanning a 30-45  $\mu\text{m}$  range. Microglia were approximately centered in the field of view when imaged. Imaris 10 software was then used to process microglia Z-stack images and generate 3D models of the soma and arborization patterns. **(A)** Microglia complexity was evaluated by determining the branch order of all ramifications present in the arborization patterns generated by the Imaris 10 algorithm; branch order can be defined as the number of ramification points from the soma taken to reach the branch being considered. Differences in the distribution of branch orders were statistically significant by two-way ANOVA ( $F_{(20, 561)} = 43.32$ ,  $p < 0.0001$ ). **(B)** Soma volume of GPR63 and GPR153 CRISPR-treated microglia were larger than NC CRISPR-treated microglia by one-way ANOVA with Tukey's post hoc test, indicative of microglia with a more amoeboid soma and a stronger microgliosis phenotype. Soma volume of GPR63 and GPR153 CRISPR-treated microglia did not statistically differ from each other, however. \*\*, \*\*\* $p < 0.01, 0.001$  vs. NC CRISPR/CIPN group. **(C)** Total length of processes emerging from the soma from GPR63 and GPR153 were significantly shorter than those of NC CRISPR-treated microglia by one-way ANOVA with Tukey's post hoc test, indicative of less ramified microglial projections and a stronger microgliosis phenotype. Total process length of GPR63 and GPR153 CRISPR-treated microglia did not

statistically differ from each other, however. \*\*\*\* $p < 0.0001$  vs. NC CRISPR/CIPN group. Data reported as mean  $\pm$  SEM. Each data point represents an individual microglia cell; N = 1 mouse per group.

### 3.4: Conclusion

In this chapter, we have shown that GPR63 and GPR153 both have detectable and widespread expression in both microglia and astrocytes, two cell types whose activity and/or dysfunction are closely tied to pathological pain states. Of particular note, GPR153 appears to be expressed in nearly all microglia which has provided the most bountiful lead to pursue our targets' potential biological functions. Taking this finding in conjunction with our behavioral screen of the impact of GPR63 and GPR153 in the context of pain and the activity of opioid drugs, we have sought to link these two receptors to biological outcomes in the spinal cord consistent with pathological pain states particularly in microglia. This approach led us to determine that the effect of CRISPR knockdown in eliminating opioid-induced antinociception in CIPN and post-surgical pain models is mediated through the function of microglia by some currently unknown mechanism. Further investigation corroborates this as we have shown that the effects of GPR63 and GPR153 CRISPR knockdown on opioid-induced antinociception can be reversed when microglia are pharmacologically inhibited, and the reduction of receptor expression appears to exacerbate microgliosis in our CIPN model. We can thus begin to speculate in more detail regarding the roles that GPR63 and GPR153 play in pathological pain states and the spinal cord as a whole. Given our findings, we believe that GPR63 and GPR153 are key regulators of microglia and act as negative

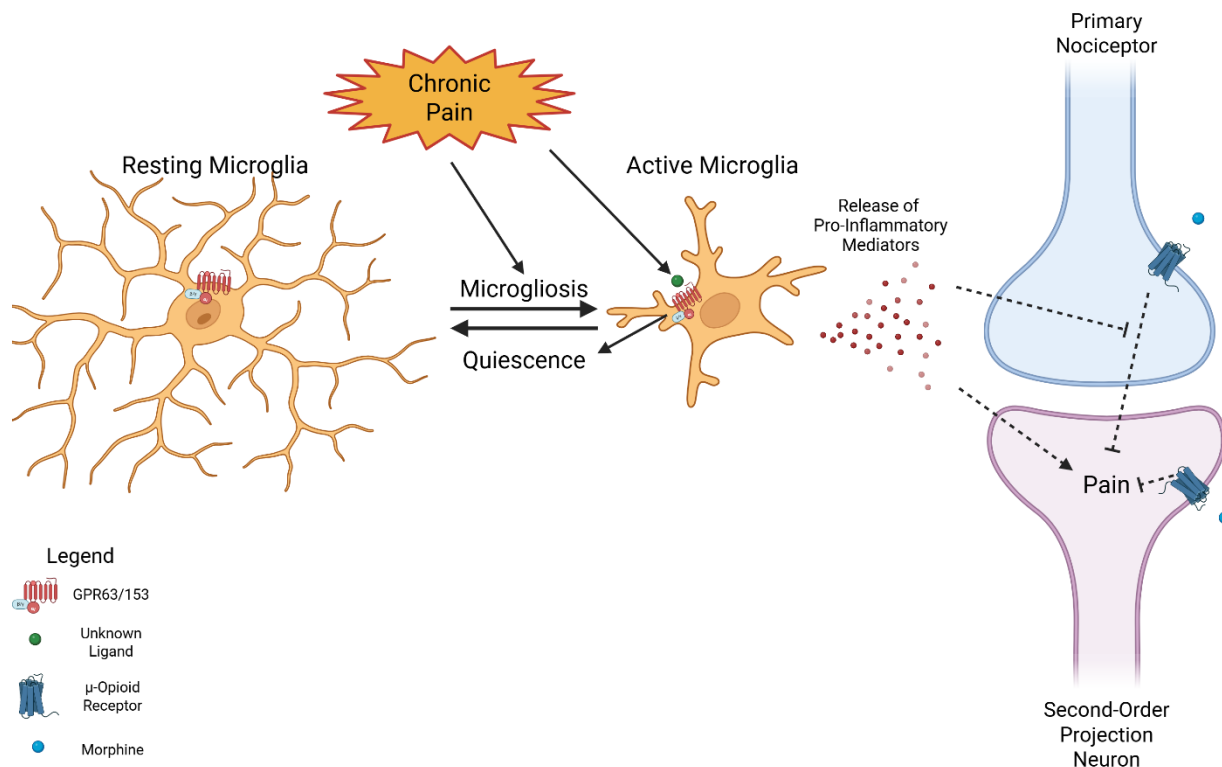
modulators of a pro-inflammatory phenotype. This hypothesis may then suggest that GPR63 and GPR153 are both likely to be  $G\alpha_i$ -coupled GPCRs and that developing agonists for these targets would be of particular therapeutic interest. We would expect agonists to effectively suppress microglial polarization during pathological pain, thus enhancing the effectiveness of pain relievers like opioids during CIPN and paw incision – and perhaps other pathological pain states. Difficulties lie, however, in that information about potential endogenous or exogenous ligands for either receptor is currently nil.

## **Chapter 4: Discussion and Future Directions**

## 4.1: Summary of Findings

To summarize, this work has identified GPR63 and GPR153 as novel targets for the modulation of microglia and opioid-induced antinociception in mouse models of pathological pain while having no impact on baseline nociception. Non-specific CRISPR knockdown of both targets in the spinal cord of adult CD-1 mice completely ablated the antinociceptive effects of morphine in a CIPN model. This effect was also observed in a model of post-surgical pain for CRISPR knockdown of GPR153 only; this treatment condition also impaired normal recovery from mechanical allodynia. Efforts to provide context to the expression of GPR63 and GPR153 in the spinal cord through RNAScope™ *in situ* hybridization concluded that both targets were expressed in at least 50% of all microglia and astrocytes in the spinal dorsal horn. In particular, nearly all microglia were found to express GPR153. Cell-specific CRISPR knockdown targeting both receptors in microglia alone replicated the findings of our non-specific knockdown experiments in both CIPN and post-surgical pain models whereas knockdown in astrocytes had no influence on behavioral outcomes in response to the pathological pain state or administration of morphine. The biological mechanism by which GPR63 and GPR153 act to prevent opioid-induced antinociception was tied to microglia further by showing that opioid-induced antinociception could be reinstated in our CRISPR-treated mice by applying the microglia inhibitor minocycline directly to the spinal cord. GPR63 and GPR153 knockdown also had notable influences on microglia morphology in a CIPN model, with CRISPR-treated microglia displaying a phenotype suggesting higher levels of microgliosis. Notably, this only seemed to occur in the context of pathological pain and not baseline nociception; this could suggest that GPR63/153 are only activated during pathological pain states, perhaps as a negative feedback loop, and presumably in response to local

release of an endogenous ligand. Together these findings inform our model: 1) GPR63 and GPR153 are expressed in microglia; 2) during pathological pain, these receptors act as a negative feedback rheostat to control the extent of microglial activation, perhaps in response to secretion of their endogenous ligands; 3) disabling GPR63/153 (as in our CRISPR experiments) superactivates microglia, while we hypothesize that GPR63/153 activation (as by agonist) would decrease microglial activity; 4) these actions of GPR63/153 then translate to decreased or increased antinociception, likely via the well-established link between microglial activity, cytokine secretion, and pain/antinociception. This model is summarized in **Figure 4.1**.



**Figure 4.1: Summary model of the proposed role of GPR63/GPR153 via microglial activity regulation in the spinal dorsal horn during pathological pain states.** GPR63/GPR153 are hypothesized to play a role in gating the progression of microgliosis in pathological pain states. This may be accomplished by the endogenous ligands either preventing microglia from becoming reactive in response to the pathological pain state or by resolving microgliosis over the course of the pathological pain state. By consequence, they likely dampen the release of pro-inflammatory cytokines from reactive microglia which are responsible for sensitizing nociceptive neurons in the spinal dorsal horn. This results in enhancement of the antinociceptive effects of opioid drugs by a currently unknown mechanism, presumably by suppressing pro-inflammatory cytokines which are known to interfere with opioid signaling.

## 4.2: Evaluation of the Therapeutic Potential of GPR63 and GPR153 and their Role in Microglial Function

Our findings that the ablation of opioid-induced antinociception correlated with GPR63 and GPR153 CRISPR knockdown and can be rescued by inhibiting microglia and that microgliosis is stronger in the presence of these knockdowns imply that GPR63 and GPR153 have inhibitory impacts on microglia. This allows us to speculate that both receptors are  $G\alpha_i$ -coupled GPCRs and the discovery/design of agonists for them could be analgesic in pathological pain conditions by further suppressing microglial activation. Inhibition of microglia has been studied as a non-opioid therapeutic target for pain relief. For instance, minocycline itself has seen investigation as a potential treatment option for CIPN with animal studies reporting that it prevents the onset of mechanical allodynia through microglia-dependent mechanisms [263; 264]. Unfortunately, assessments of therapeutic viability in human cancer patients have not shown minocycline treatment to be effective in preventing or relieving symptoms of CIPN [265-267]. Microglia-related inflammation contributing to pathological pain can be targeted by other drug classes such as antibody therapies targeting proinflammatory mediators (e.g., IL-1 $\beta$ , TREM2) with some showing efficacy in arthritic cases [268-270]. Drugs such as these, however, do not remedy the presence of microgliosis but rather just attempt to mitigate the products of it which may not be a sufficient approach for advancing the resolution of pathological pain. Our proposed hypothesis that GPR63 and GPR153 agonists could shift microglia into a quiescent state in cases of pathological pain would address this issue and potentially promote resolution of pain. Similar to this line of thought, other mechanisms of microglial activation such as TLR4 stimulation have

been targeted clinically using antagonists that demonstrate effectiveness in reducing inflammatory cytokine production in osteoarthritis patients, but efficacy in yielding analgesia is yet to be clarified [271]. Another contention with the use of minocycline as a microglial inhibitor lies in that it is a tetracycline antibiotic with multiple known mechanisms in which it produces anti-inflammatory outcomes rather than specifically targeting microglia, and this introduces the risk of our observations being driven by a combination of off-target effects [252]. Other alternatives for specifically targeting microglia are possible, however, through pharmacological interventions such as CSF1R inhibitors or the use of targeted chemogenetic inhibition via DREADDs [223; 232; 272]. CSF1R inhibitors still present the drawback of potential influences beyond the CNS and off-target effects but offer more specificity in targeting microglia compared to minocycline [273].

Although illuminating and pioneering, this work is not entirely comprehensive in the investigation of GPR63 and GPR153 in the context of microglial biology and allows for several other avenues of investigation. Neuroinflammation is a hallmark of numerous CNS disease states as discussed in previous chapters, and much of this is driven by the release of inflammatory cytokines from active microglia. The effects of GPR63 and GPR153 knockdown or overexpression on cytokine expression and release were not evaluated in this study, however. Based on our results, we can hypothesize that knockdown may result in increases in expression and release whereas overexpression may trend in the opposite direction to mitigate expression and release of cytokines. Future investigation of these two receptors in microglia can evaluate these outcomes through numerous approaches such as RT-qPCR or ELISA analysis of whole spinal cord tissue from GPR63 and GPR153 CRISPR-treated mice or *in vitro* assessments using cultured human and/or mouse primary microglia. The role of GPR63 and GPR153 outside of pathological pain may also

be of interest to develop a stronger understanding of the role of microglia in healthy tissue. Like our morphology approach in the CIPN condition, we can similarly assess microglia morphology in healthy/sham-treated conditions in the presence and absence of GPR63 and GPR153 CRISPR knockdown. It is possible that knockdown of both receptors also results in a more pro-inflammatory phenotype in the absence of a pathological insult which would imply that GPR63 and GPR153 signaling acts endogenously as a gating mechanism for microgliosis. These outcomes could also be evaluated and possibly treated *in vivo* following pharmacological manipulation (i.e., administration of GPR63 and GPR153 agonists); this option is not currently available but may be soon through our lab's ligand screening efforts described below.

We observed no impacts of GPR63 and GPR153 CRISPR knockdown on acute thermal nociception or baseline mechanical nociception in our tail flick and CIPN paradigms, respectively. This finding is intriguing in conjunction with our hypothesis that these receptors suppress microglial activation as microglial activation alone can induce pain-like behaviors in the absence of a pathological state [274]. Our only current explanation for this lies in speculating that endogenous activity of these receptors (i.e., ligand expression and release) is not present under healthy circumstances. Although we show that both GPR63 and GPR153 are notably expressed in naïve mouse spinal cord tissue, it is not indicative of receptor activity. This work could further suggest that GPR63 and GPR153 endogenous ligands are released during pathological pain states like CIPN.

Microglia have been demonstrated to affect the resolution of pathological pain states if they present an anti-inflammatory phenotype [221; 222]. Depletion and repopulation of microglia in an injury model also demonstrates that repopulated microglia have different transcriptomic profiles

compared to those present at the onset of pathological pain which may contribute to pain resolution [223]. Considering these outcomes and our data regarding GPR153 CRISPR knockdown impairing recovery in our post-surgical pain model, we can speculate that GPR153 may be a crucial mediator for pain resolution. Our findings that GPR153 is expressed in nearly all microglia may indicate that this receptor has greater leverage within the context of gating microgliosis and could be identified as the more effective target compared to GPR63 with further investigation. An explanation as to why we were able to observe persistent mechanical allodynia despite opioid administration with knockdown of both targets may also imply that CIPN is more sensitive to microglial activation relative to persistent post-surgical pain. Both observations in CIPN and post-surgical pain may have something to do with the interplay between microglia and astrocytes in pathological pain states involving neuroinflammation as some cases report microglial reactivity preceding astrogliosis [225; 227; 228].

Our work also implicates GPR63 and GPR153 in the microglial modulation of endogenous opioid signaling because of the specific loss of opioid-induced antinociception in our behavioral models without any effects in baseline measurements. It is unclear if this outcome is caused by complete silencing of opioidergic signaling mechanisms in the spinal cord following GPR63 and GPR153 CRISPR knockdown and suspected hyperactivity of microglia. Conversely, opioidergic signaling may simply be dampened under these experimental conditions, and the dose of morphine administered was insufficient. Reports of microglial reactivity contributing to opioid tolerance and reductions in analgesic efficacy support this theory [195; 275]. This could potentially be addressed through a dose-response approach to evaluate if higher doses of morphine are capable of eliciting any antinociception. From a neurocircuit perspective, reactive microglia have also been shown to

impact endogenous opioid signaling with correlations to decreased descending modulation from the PAG-RVM pathways while increasing glutamatergic tone in the spinal dorsal horn [276; 277]. Hyperalgesic priming following opioid administration has been linked to TLR4 as well, a key mediator of microglial activation and neuroinflammation as a whole [10; 278; 279]. Other adaptations of our current behavioral experiments could be introduced as well to investigate the effects of GPR63 and GPR153 knockdown on opioid-induced hyperalgesia and tolerance by monitoring opioid-induced antinociception following target knockdown and repeated opioid administration.

### 4.3: Potential Roles of GPR63 and GPR153 in Other Cell Types of the Spinal Cord and Endogenous Opioid Signaling

Both GPR63 and GPR153 have been confirmed to have expression in both microglia and astrocytes within the mouse spinal cord as demonstrated by the colocalization data presented in **Section 3.3.1**. Our behavioral data employing cell-specific CRISPR knockdown of GPR63 and GPR153 only implicated their impact on pathological pain states when targeted to microglia, however. Despite this finding, it is unlikely that both targets would have no effect on astrocyte function; the context of our experiments may have just not been suitable to distinguish this. The hypothesis that these targets act as inhibitory GPCRs would lead us to believe that their activity could also inhibit the development of astrogliosis. Data supporting this hypothesis would be crucial as astrocyte reactivity has been reported to contribute to the development of CIPN with multiple chemotherapy agents [31; 235]. Several measures of astrogliosis exist in the literature such as

evaluating the formation of glial scars following spinal cord injury, their responsiveness to inflammatory cytokines, or their morphology [280; 281]. The interplay between microglia and astrocytes is also heavily studied in neuroinflammatory contexts which would encourage examining the effects of GPR63 and GPR153 knockdown on their interactions via *in vitro* co-culture of the two cell types [282].

Although not specifically interrogated in our studies, it is highly likely that GPR63 and GPR153 are also expressed on neurons in the spinal cord; this can be inferred by the clear and widespread expression of both targets throughout the gray matter of the spinal dorsal horn. Support for this also comes from one spatial transcriptomic study of human DRGs reporting GPR153 expression on putative silent nociceptors [145]. Silent nociceptors themselves are particularly interesting in the context of our findings as they are characterized as insensitive to nociceptive stimuli under normal conditions but become active following sensitization by pro-inflammatory mediators [283; 284]. Investigating GPR63 and GPR153 would prove to be a more complex endeavor compared to microglia and astrocytes considering the presence of both excitatory and inhibitory neurons distributed throughout the nociceptive circuit. Following the hypothesis that GPR63 and GPR153 are both inhibitory GPCRs in combination with our behavioral results allows us to speculate that they may be expressed on pro-nociceptive neurons. CRISPR knockdown of both receptors *in vivo* suggests that more nociceptive signaling may be occurring which may be a result of disinhibition of nociceptive neurons or associated facilitatory interneurons. Colocalization studies and cell-specific knockdown approaches similar to what has been performed here using markers for nociceptors (e.g., CGRP) or excitatory neurons (e.g., vesicular glutamate transporter 2, vGluT2; LIM homeobox transcription factor 1-beta; Lmx1b) could

demonstrate comparable impacts on mitigating opioid-induced antinociception by increasing the firing tone of nociceptive signals [285]. Using pan-neuronal markers such as NeuN could provide some insights but would likely be limited because of its inability to distinguish neuronal subtypes. Studying these receptors in neurons also opens opportunities for a wide array of physiological assessments such as patch-clamp electrophysiology, measurement of evoked neurotransmitter release, or  $\text{Ca}^{2+}$  imaging to determine their influence on neuronal excitability. Some of these experiments would likely require the discovery of a ligand to be most viable, however.

#### 4.4: Ligand Screening and Deorphanization Efforts

Presently, no reliable ligands for GPR63 and GPR153 have been reported. Sphingosine-1-phosphate (S1P) has been reported as a low affinity agonist for GPR63 [164; 286; 287]. These reports suggested that GPR63 may have some role in pain modulation as S1P signaling has been under investigation in the search for non-opioid analgesics, albeit as a pro-nociceptive molecule [165]. We have been unable to demonstrate any agonist activity by S1P at GPR63 using a PRESTO-Tango assay (**data not shown**), however, along with another group similarly unable to replicate these results [288]. No reports of any potential ligands for GPR153 are currently available. Nevertheless, sequence phylogeny analysis of GPR63 and GPR153 may provide some leads in regards to potential ligands. GPR153 has been demonstrated to have sequence similarity with members of the adrenergic and serotonergic receptor families which are both known to modulate nociception in some capacity [144; 289]. Similarly, GPR63 shares lineage with numerous receptors for lipid mediators such as the cannabinoid receptors which are also implicated

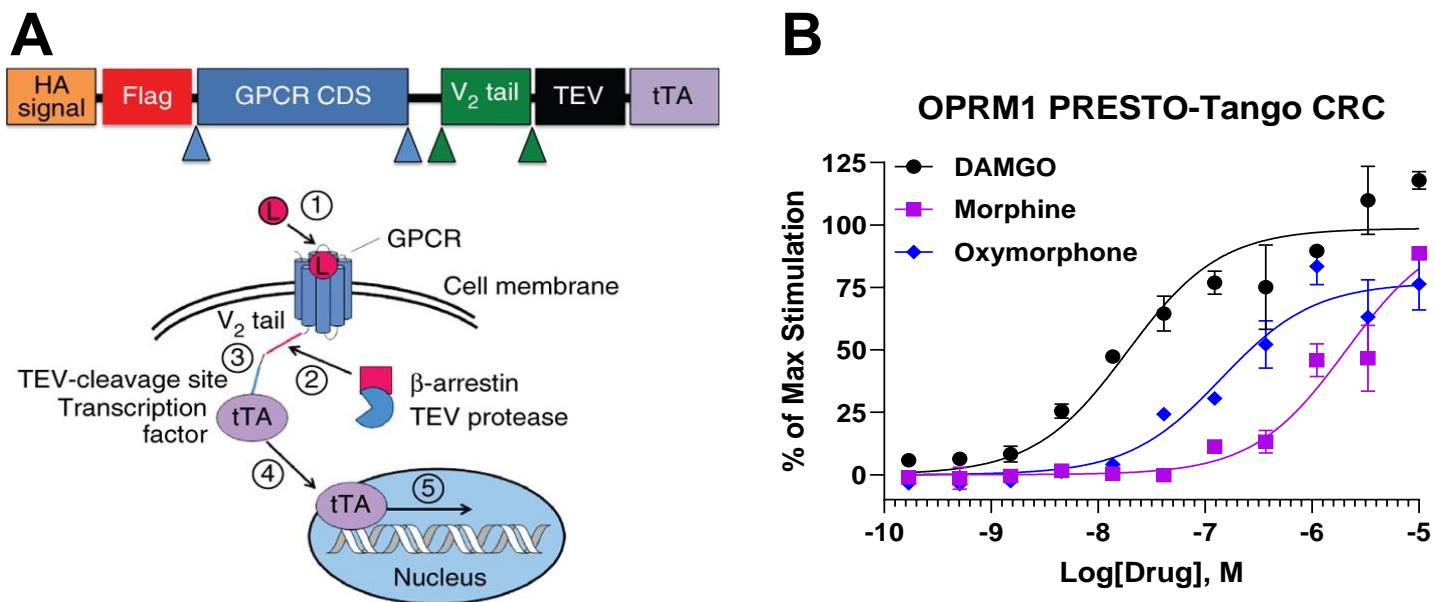
in pain modulation [146]. Although promising, the gaps in concrete knowledge surrounding our targets complicates the process of deorphanization and identifying their biological influences. Furthermore, these receptors being full orphans rules out any knowledge of their G-protein coupling and prevents us from performing screening experiments by conventional means of measuring cAMP production ( $G\alpha_s$ ,  $G\alpha_i$ ) or intracellular  $Ca^{2+}$  concentrations ( $G\alpha_q$ ).

Fortunately, GPCR activity is not strictly measurable by G-protein signaling pathways, and pharmacologists have engineered novel assays in recent years to interrogate GPCR activation for screening purposes. Of particular interest for us has been the Tango assay developed by Dr. Gilad Barnea's group and its modularization and open-source access made possible through the PRESTO-Tango adaptation created by Dr. Bryan Roth's group [290; 291]. The PRESTO-Tango (Parallel Receptor-ome Expression and Screening via Transcription Output – Tango) assay capitalizes on the recruitment of  $\beta$ -arrestin as another intracellular signaling pathway indicative of GPCR stimulation (**Fig. 4.2A**). In this assay, two genetically engineered components are unified to generate a measurable output: (1) a cell line containing a modified  $\beta$ -arrestin protein bound to a tobacco etch virus (TEV) protease along with a luciferase reporter gene whose transcription is driven by a tetracycline transactivator (tTA) transcription factor and (2) a modified GPCR of interest in which the C-terminus is conjugated to a tTA transcription factor by a short amino acid sequence containing a TEV protease cleavage site. Upon ligand binding and subsequent  $\beta$ -arrestin recruitment to the GPCR's intracellular domains allows for the TEV protease to release the tTA transcription factor which translocates to the nucleus and induces transcription of the reporter gene. We have confirmed the viability of this assay in our lab using the PRESTO-Tango construct for the MOR showing appropriate responses to the ligands DAMGO, morphine, and oxymorphone

(**Fig. 4.2B**). By this method, we are currently performing high-throughput screening of various drug and natural product libraries to be able to probe our targets pharmacologically in the near future. These efforts are also joined by work with our collaborators using *in silico* modeling to construct peptide and small molecule structures that interact favorably with the proposed binding pockets of GPR63 and GPR153.

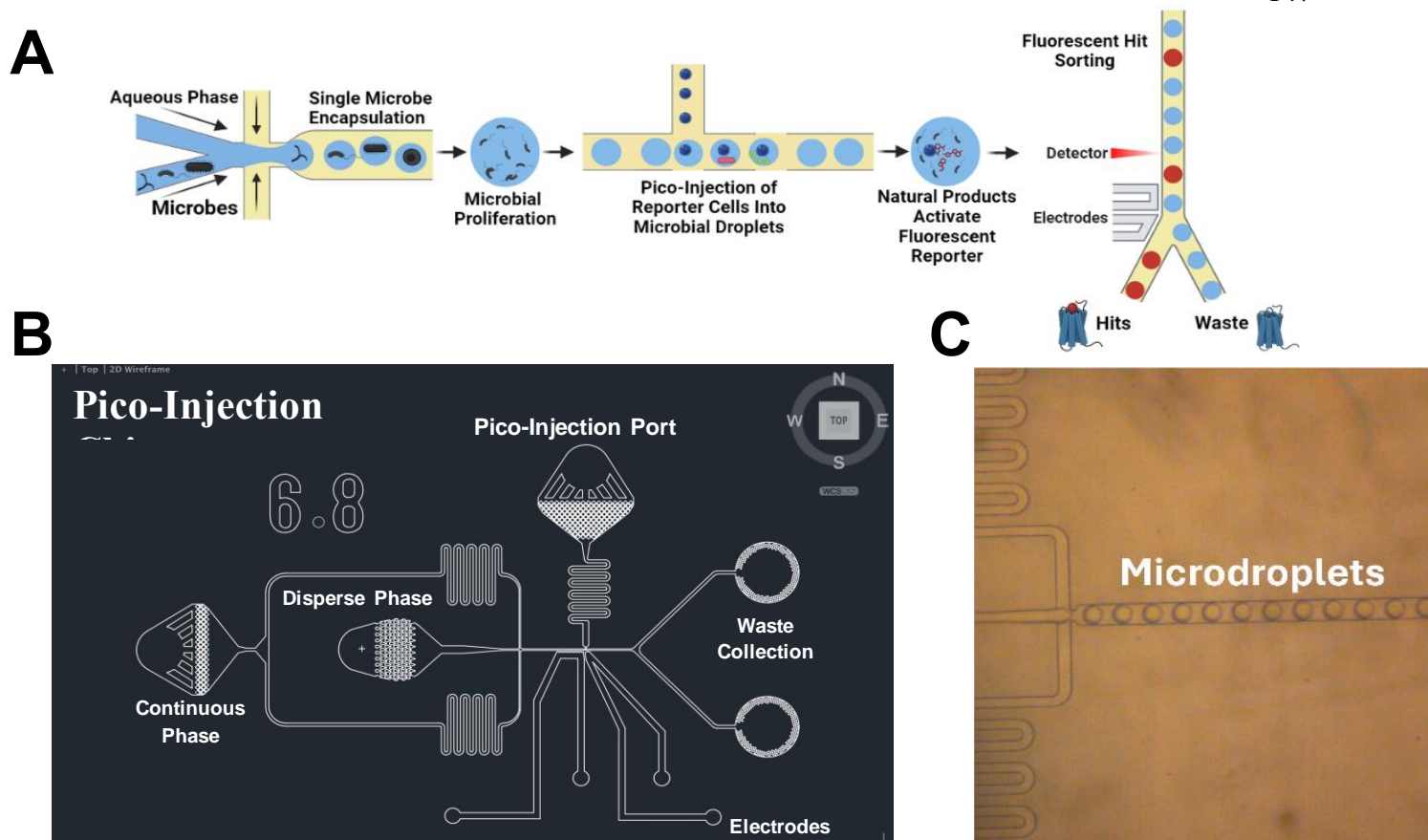
Natural products are a tremendous resource for pharmacological discovery as evidenced by opioids and cannabinoids, two classes of analgesic drugs derived from plants and used medicinally throughout the course of history. As such, it is vital to consider diverse natural product reservoirs in our endeavors to deorphanize GPR63 and GPR153. One such reservoir lies in microbes. Numerous drugs instrumental for driving medical breakthroughs have been derived from bacteria such as antibiotics and chemotherapy agents [292-294]. Through our lab's collaboration with Dr. Christopher Cartmell, we are actively developing a state-of-the-art high-throughput microfluidics screening platform to target microbially derived natural products against the GPR63/GPR153 PRESTO-Tango assay. The platform operates by encapsulating individual microbes in microdroplets followed by pico-injection of individual reporter cells expressing the PRESTO-Tango construct for our targets of interest (**Fig. 4.3A**). Microbes can then be allowed to proliferate alongside reporter cells within their isolated microdroplet environments such that any natural products generated by collected microbes can interact with the GPR63/GPR153 PRESTO-Tango receptors. The PRESTO-Tango assay itself can be modified to exchange the current bioluminescent reporter for a fluorescent reporter, and microdroplets containing hits for receptor activation can be separated by fluorescence-activated droplet sorting (FADS) [295]. Positive hits for receptor activation would then undergo biochemical analysis to identify the specific natural

product responsible with potential room for medicinal chemistry approaches to optimize ligand-receptor interactions and/or receptor stimulation. Interrogation of the downstream signaling pathways of GPR63 and GPR153 would also become feasible, contributing further insights into their biological roles and therapeutic potential. Development of this assay has been successful thus far through our proven ability to form microdroplets (**Fig. 4.3C**) and encapsulate individual bacteria and mammalian cells within them (data not shown).



**Figure 4.2: The PRESTO-Tango assay for identifying GPCR activation via  $\beta$ -arrestin recruitment.** (A) The PRESTO-Tango assay is an open-source platform designed for screening orphan ligands and/or GPCRs. A specially engineered HTLA cell line expressing a tTA-dependent luciferase reporter is transfected with an expression construct for a genetically engineered variant of the GPCR of interest. This GPCR is unique in that it is conjugated to a tTA transcription factor by a short sequence containing a TEV cleavage site. The HTLA cell line itself also expresses a genetically modified variant of  $\beta$ -arrestin conjugated to TEV protease. Upon receptor activation due to ligand binding,  $\beta$ -arrestin is recruited to the C-terminus of the GPCR target and the TEV protease cleavage activity releases the tTA transcription factor. The transcription factor then translocates to the nucleus and induces expression of the luciferase reporter gene thus allowing GPCR activation to be measured via bioluminescence. Figure adapted from [291]. (B) Proof-of-concept of the PRESTO-Tango assay's viability in our hands. The PRESTO-Tango construct for the  $\mu$ -opioid receptor (MOR/*Oprm1*) was transfected into HTLA cells obtained from the lab of Gilad Barnea, Ph.D. at Brown University. Cells were then stimulated with known MOR agonists

DAMGO, morphine, and oxymorphone. All three ligands were efficacious in stimulating  $\beta$ -arrestin recruitment with DAMGO providing the highest efficacy and potency consistent with its identity as a highly selective MOR agonist. Data collected by John M. Streicher, Ph.D. and reported as mean  $\pm$  SEM.



**Figure 4.3: A microfluidics platform for high-throughput screening of natural product ligands.** (A) Workflow of the high-throughput microfluidic natural product screening platform for GPR63 and GPR153 drug discovery. Individual microbes are encapsulated in microdroplets and allowed to proliferate over time and perform their normal biological processes resulting in the presence of chemical products serving as potential ligands. Microdroplets with encapsulated microbes are then pico-injected with individual mammalian fluorescent reporter cells expressing the PRESTO-Tango construct of either GPR63 or GPR153. Microdroplets in which GPR63 or GPR153 are stimulated by natural products generated by their microbial neighbors are then sorted by FADS and biochemical analysis of the natural products found in the microdroplets containing positive hits will be performed. (B) Schematic diagram of the microfluidics chip being used to generate microdroplets, encapsulate microbes, and perform pico-injection of reporter cells. (C)

Proof-of-concept of the successful generation of microdroplets in the microfluidics chip. All figures provided by Christopher Cartmell, Ph.D.

## 4.5: Closing Remarks

The studies presented here have established the scientific premise for investigation of GPR63 and GPR153 as novel non-opioid modulators of pathological pain states, an ever-growing and critical public health need. The nature of GPR63 and GPR153 as full orphan receptors itself warrants further study from the standpoint of the raw potential for scientific discovery particularly in the CNS. The effects of targeting these receptors appear to be mediated by the activity of microglia which presents a cornucopia of possible avenues of investigation for implicating these targets in pathological pain, numerous other CNS disease states, and fundamental questions of neurobiology and neuropharmacology. Future scientific evaluation and deorphanization of these receptors is spearheaded by the active search for a ligand which would initiate the process of evaluating the therapeutic viability of GPR63 and GPR153 as drug targets. In conclusion, GPR63 and GPR153 are promising novel targets for studying pathological pain states, but their place within the study of pain is yet to be fully determined.

## References

- [1] Lucas, J. W., Sohi, I. Chronic Pain and High-impact Chronic Pain in U.S. Adults, 2023. NCHS Data Brief 2024(518).
- [2] Gaskin, D. J., Richard, P. The economic costs of pain in the United States. *J Pain* 2012;13(8):715-724.
- [3] De La Rosa, J. S., Brady, B. R., Ibrahim, M. M., Herder, K. E., Wallace, J. S., Padilla, A. R., Vanderah, T. W. Co-occurrence of chronic pain and anxiety/depression symptoms in U.S. adults: prevalence, functional impacts, and opportunities. *Pain* 2024;165(3):666-673.
- [4] De La Rosa, J. S., Brady, B. R., Herder, K. E., Wallace, J. S., Ibrahim, M. M., Allen, A. M., Meyerson, B. E., Suhr, K. A., Vanderah, T. W. The unmet mental health needs of U.S. adults living with chronic pain. *Pain* 2024;165(12):2877-2887.
- [5] Raja, Srinivasa N, Carr, Daniel B, Cohen, Milton, Finnerup, Nanna B, Flor, Herta, Gibson, Stephen, Keefe, Francis, Mogil, Jeffrey S, Ringkamp, Matthias, Sluka, Kathleen A, Song, Xue-Jun, Stevens, Bonnie, Sullivan, Mark, Tutelman, Perri, Ushida, Takahiro, Vader, Kyle. The Revised IASP definition of pain: concepts, challenges, and compromises. *Pain* 2020 Sep 1;161(9).
- [6] Finnerup, Nanna Brix, Kuner, Rohini, Jensen, Troels Staehelin. Neuropathic Pain: From Mechanisms to Treatment. *Physiological Reviews* 2020 Nov 03;101(1).
- [7] Zajączkowska, Renata, Kocot-Kępska, Magdalena, Leppert, Wojciech, Wrzosek, Anna, Mika, Joanna, Wordliczek, Jerzy. Mechanisms of Chemotherapy-Induced Peripheral Neuropathy. *International Journal of Molecular Sciences* 2019 Mar 22;20(6).
- [8] Neil, Michael JE, Macrae, William A. Post Surgical Pain- The Transition from Acute to Chronic Pain. *Reviews in Pain* 2009 Oct;3(2).
- [9] A, Miclescu, A, Straatmann, P, Gkatziani, S, Butler, R, Karlsten, T, Gordh. Chronic neuropathic pain after traumatic peripheral nerve injuries in the upper extremity: prevalence, demographic and surgical determinants, impact on health and on pain medication - PubMed. *Scandinavian journal of pain* 12/18/2019;20(1).
- [10] M, Echeverria-Villalobos, V, Tortorici, BE, Brito, D, Ryskamp, A, Uribe, T, Weaver. The role of neuroinflammation in the transition of acute to chronic pain and the opioid-induced hyperalgesia and tolerance - PubMed. *Frontiers in pharmacology* 12/15/2023;14.
- [11] Shokri, Pourya, Zahmatyar, Mahdi, Tafti, Mahdi Falah, Fathy, Mobin, Tolzali, Mohammadmahdi Rezaei, Jolfayi, Amir Ghaffari, Nejadghaderi, Seyed Aria, Sullman, Mark J M, Kolahi, Ali-Asghar, Safiri, Saeid. Non-spinal low back pain: Global epidemiology, trends, and risk factors. *Health Science Reports* 2023 Sep 4;6(9).
- [12] R, Siracusa, RD, Paola, S, Cuzzocrea, D, Impellizzeri. Fibromyalgia: Pathogenesis, Mechanisms, Diagnosis and Treatment Options Update - PubMed. *International journal of molecular sciences* 04/09/2021;22(8).
- [13] TS, Jensen, NB, Finnerup. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms - PubMed. *The Lancet Neurology* 2014 Sep;13(9).
- [14] J, Sandkühler. Models and mechanisms of hyperalgesia and allodynia - PubMed. *Physiological reviews* 2009 Apr;89(2).

- [15] Gabriel, K. A., Streicher, J. M. HSP90 inhibition in the mouse spinal cord enhances opioid signaling by suppressing an AMPK-mediated negative feedback loop. *Sci Signal* 2023;16(780):eade2438.
- [16] Lei, Wei, Mullen, Nathan, McCarthy, Sarah, Brann, Courtney, Richard, Philomena, Cormier, James, Edwards, Katie, Bilsky, Edward J., Streicher, John M. Heat-shock protein 90 (Hsp90) promotes opioid-induced anti-nociception by an ERK mitogen-activated protein kinase (MAPK) mechanism in mouse brain. *Journal of Biological Chemistry* 2017/06/23;292(25).
- [17] AM, Schwarz, A, Keresztes, T, Bui, R, Hecksel, A, Peña, B, Lent, ZG, Gao, M, Gamez-Rivera, CA, Seekins, K, Chou, TL, Appel, KA, Jacobson, FA, Al-Obeidi, JM, Streicher. Terpenes from *Cannabis sativa* induce antinociception in a mouse model of chronic neuropathic pain via activation of adenosine A<sub>2A</sub> receptors - PubMed. *Pain* 11/01/2024;165(11).
- [18] DI, Duron, W, Lei, NK, Barker, C, Stine, S, Mishra, BSJ, Blagg, PR, Langlais, JM, Streicher. Inhibition of Hsp90 in the spinal cord enhances the antinociceptive effects of morphine by activating an ERK-RSK pathway - PubMed. *Science signaling* 05/05/2020;13(630).
- [19] Cowie, Ashley, Stucky, Cheryl. A Mouse Model of Postoperative Pain. *BIO-PROTOCOL* 2019;9(2).
- [20] Fuller, Alice M., Bharde, Sabah, Sikandar, Shafaq. Frontiers | The mechanisms and management of persistent postsurgical pain. *Frontiers in Pain Research* 2023/07/06;4.
- [21] G, Wolf, D, Livshits, B, Beilin, R, Yirmiya, Y, Shavit. Interleukin-1 signaling is required for induction and maintenance of postoperative incisional pain: genetic and pharmacological studies in mice - PubMed. *Brain, behavior, and immunity* 2008 Oct;22(7).
- [22] EU, Carreira, V, Carregaro, MM, Teixeira, A, Moriconi, A, Aramini, WA, Verri, SH, Ferreira, FQ, Cunha, TM, Cunha. Neutrophils recruited by CXCR1/2 signalling mediate post-incisional pain - PubMed. *European journal of pain (London, England)* 2013 May;17(5).
- [23] RK, Banik, TJ, Brennan. Spontaneous discharge and increased heat sensitivity of rat C-fiber nociceptors are present in vitro after plantar incision - PubMed. *Pain* 2004 Nov;112(1-2).
- [24] HJ, Lee, EM, Pogatzki-Zahn, TJ, Brennan. The effect of the AMPA/kainate receptor antagonist LY293558 in a rat model of postoperative pain - PubMed. *The journal of pain* 2006 Oct;7(10).
- [25] Y, Wang, J, Wu, R, Guo, Y, Zhao, Y, Wang, M, Zhang, Z, Chen, A, Wu, Y, Yue. Surgical incision induces phosphorylation of AMPA receptor GluR1 subunits at Serine-831 sites and GluR1 trafficking in spinal cord dorsal horn via a protein kinase C $\gamma$ -dependent mechanism - PubMed. *Neuroscience* 06/14/2013;240.
- [26] C, Liu, S, Luan, H, OuYang, Z, Huang, S, Wu, C, Ma, J, Wei, W, Xin. Upregulation of CCL2 via ATF3/c-Jun interaction mediated the Bortezomib-induced peripheral neuropathy - PubMed. *Brain, behavior, and immunity* 2016 Mar;53.
- [27] Canta, Annalisa, Pozzi, Eleonora, Carozzi, Valentina Alda. Mitochondrial Dysfunction in Chemotherapy-Induced Peripheral Neuropathy (CIPN). *Toxics* 2015 Jun 5;3(2).
- [28] Scripture, Charity D, Figg, William D, Sparreboom, Alex. Peripheral Neuropathy Induced by Paclitaxel: Recent Insights and Future Perspectives. *Current Neuropharmacology* 2006 Apr;4(2).
- [29] M, Weintraub, MA, Adde, DJ, Venzon, AT, Shad, ID, Horak, JE, Neely, NL, Seibel, J, Gootenberg, C, Arndt, ML, Nieder, IT, Magrath. Severe atypical neuropathy associated

- with administration of hematopoietic colony-stimulating factors and vincristine - PubMed. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 1996 Mar;14(3).
- [30] Boyette-Davis, J, Xin, W, Zhang, H, Dougherty, PM. Intraepidermal nerve fiber loss corresponds to the development of Taxol-induced hyperalgesia and can be prevented by treatment with minocycline. *Pain* 2010 Dec 9;152(2).
- [31] Zhang, Haijun, Yoon, Seo-Yeon, Zhang, Hongmei, Dougherty, Patrick M. Evidence that spinal astrocytes but not microglia contribute to the pathogenesis of paclitaxel-induced painful neuropathy. *The Journal of Pain* 2012 Jan 29;13(3).
- [32] J, Ruiz-Medina, A, Baulies, SA, Bura, O, Valverde. Paclitaxel-induced neuropathic pain is age dependent and devolves on glial response - PubMed. *European journal of pain (London, England)* 2013 Jan;17(1).
- [33] O'Brien, Phillippe D, Sakowski, Stacey A, Feldman, Eva L. Mouse Models of Diabetic Neuropathy. *ILAR Journal* 2014;54(3).
- [34] HE, Korah, K, Cheng, SM, Washington, ME, Flowers, HJ, Stratton, A, Patwardhan, MM, Ibrahim, LF, Martin. Partial Sciatic Nerve Ligation: A Mouse Model of Chronic Neuropathic Pain to Study the Antinociceptive Effect of Novel Therapies - PubMed. *Journal of visualized experiments : JoVE* 10/06/2022(188).
- [35] Noh, Ain' Sabreena Mohd, Chuan, Tan Dai, Khir, Nurul Ajilah Mohamed, Zin, Anani Aila Mat, Ghazali, Anis Kausar, Long, Idris, Aziz, Che Badariah Ab, Ismail, Che Aishah Nazariah. Effects of different doses of complete Freund's adjuvant on nociceptive behaviour and inflammatory parameters in polyarthritic rat model mimicking rheumatoid arthritis. *PLoS ONE* 2021 Dec 8;16(12).
- [36] Catorce, Miryam Nava, Gevorkian, Goar. LPS-induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-clinical Assessment of Nutraceuticals. *Current Neuropharmacology* 2016 Feb;14(2).
- [37] Treede, Rolf-Detlef, Rief, Winfried, Barke, Antonia, Aziz, Qasim, Bennett, Michael I., Benoliel, Rafael, Cohen, Milton, Evers, Stefan, Finnerup, Nanna B., First, Michael B., Giamberardino, Maria Adele, Kaasa, Stein, Korwisi, Beatrice, Kosek, Eva, Lavand'homme, Patricia, Nicholas, Michael, Perrot, Serge, Scholz, Joachim, Schug, Stephan, Smith, Blair H., Svensson, Peter, Vlaeyen, Johan W.S., Wang, Shuu-Jiun. Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). *PAIN* January 2019;160(1).
- [38] Scopus - Document details - Amiloride-blockable acid-sensing ion channels are leading acid sensors expressed in human nociceptors | Signed in. *Journal of Clinical Investigation* 2002;110(8).
- [39] KJ, Bär, G, Natura, A, Telleria-Diaz, P, Teschner, R, Vogel, E, Vasquez, HG, Schaible, A, Ebersberger. Changes in the effect of spinal prostaglandin E2 during inflammation: prostaglandin E (EP1-EP4) receptors in spinal nociceptive processing of input from the normal or inflamed knee joint - PubMed. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 01/21/2004;24(3).
- [40] AM, Binshtok, H, Wang, K, Zimmermann, F, Amaya, D, Vardeh, L, Shi, GJ, Brenner, RR, Ji, BP, Bean, CJ, Woolf, TA, Samad. Nociceptors are interleukin-1beta sensors - PubMed. *The*

- Journal of neuroscience : the official journal of the Society for Neuroscience 12/24/2008;28(52).
- [41] Zeitz, Karla P, Guy, Nicolas, Malmberg, Annika B, Dirajlal, Sahera, Martin, William J, Sun, Linda, Bonhaus, Douglas W, Stucky, Cheryl L, Julius, David, Basbaum, Allan I. The 5-HT3 Subtype of Serotonin Receptor Contributes to Nociceptive Processing via a Novel Subset of Myelinated and Unmyelinated Nociceptors. *The Journal of Neuroscience* 2002 Feb 1;22(3).
- [42] Dubin, Adrienne E, Patapoutian, Ardem. Nociceptors: the sensors of the pain pathway. *The Journal of Clinical Investigation* 2010 Nov 1;120(11).
- [43] A, Dhaka, AN, Murray, J, Mathur, TJ, Earley, MJ, Petrus, A, Patapoutian. TRPM8 is required for cold sensation in mice - PubMed. *Neuron* 05/03/2007;54(3).
- [44] MJ, Caterina, MA, Schumacher, M, Tominaga, TA, Rosen, JD, Levine, D, Julius. The capsaicin receptor: a heat-activated ion channel in the pain pathway - PubMed. *Nature* 10/23/1997;389(6653).
- [45] DM, Bautista, J, Siemens, JM, Glazer, PR, Tsuruda, AI, Basbaum, CL, Stucky, SE, Jordt, D, Julius. The menthol receptor TRPM8 is the principal detector of environmental cold - PubMed. *Nature* 07/12/2007;448(7150).
- [46] Coste, Bertrand, Mathur, Jayanti, Schmidt, Manuela, Earley, Taryn J, Ranade, Sanjeev, Petrus, Matt J, Dubin, Adrienne E, Patapoutian, Ardem. Piezo1 and Piezo2 are essential components of distinct mechanically-activated cation channels. *Science (New York, NY)* 2010 Sep 2;330(6000).
- [47] Haberberger, Rainer Viktor, Barry, Christine, Dominguez, Nicholas, Matusica, Dusan. Human Dorsal Root Ganglia. *Frontiers in Cellular Neuroscience* 2019 Jun 19;13.
- [48] Kandel, Eric, Schwartz, James, Jessell, Thomas, Jessell, Department of Biochemistry, Molecular Biophysics, Thomas, Siegelbaum, Steven, Hudspeth, A. J. *Principles of Neural Science*, Fifth Edition.
- [49] MB, MacIver, DL, Tanelian. Structural and functional specialization of A delta and C fiber free nerve endings innervating rabbit corneal epithelium - PubMed. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1993 Oct;13(10).
- [50] Hees, J Van, Gybels, J. C nociceptor activity in human nerve during painful and non painful skin stimulation. *Journal of Neurology, Neurosurgery, and Psychiatry* 1981 Jul;44(7).
- [51] Todd, Andrew J. *Nociceptive Circuitry in the Spinal Cord*. Encyclopedia of Pain 2013.
- [52] Yam, Mun Fei, Loh, Yean Chun, Tan, Chu Shan, Adam, Siti Khadijah, Manan, Nizar Abdul, Basir, Rusliza. General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain Regulation. *International Journal of Molecular Sciences* 2018 Jul 24;19(8).
- [53] Loseth, G. E., Ellingson, D., & Leknes, S. *Touch and pain*. Noba textbook series: Psychology. Champaign, IL: DEF Publishers, 2025.
- [54] Derderian C, Shumway KR, Tadi P. *Physiology, Withdrawal Response*. StatPearls. Treasure Island (FL): StatPearls Publishing, 2023.
- [55] Apkarian, A. Vania. *Nociceptive Processing in the Thalamus*. 2007.
- [56] Sun, Guanghao, McCartin, Michael, Liu, Weizhuo, Zhang, Qiaosheng, Kenefati, George, Chen, Zhe Sage, Wang, Jing, Sun, Guanghao, McCartin, Michael, Liu, Weizhuo, Zhang, Qiaosheng, Kenefati, George, Chen, Zhe Sage, Wang, Jing. *Temporal pain processing in*

- the primary somatosensory cortex and anterior cingulate cortex. *Molecular Brain* 2023 16:1 2023-01-05;16(1).
- [57] Pain Affect Encoded in Human Anterior Cingulate But Not Somatosensory Cortex. *Science* 1997;277(5328).
- [58] Labrakakis, Charalampos. The Role of the Insular Cortex in Pain. *International Journal of Molecular Sciences* 2023 Mar 17;24(6).
- [59] Neugebauer, Volker. Amygdala physiology in pain. *Handbook of behavioral neuroscience* 2020 Mar 31;26.
- [60] Ong, Wei-Yi, Stohler, Christian S, Herr, Deron R. Role of the Prefrontal Cortex in Pain Processing. *Molecular Neurobiology* 2018 Jun 6;56(2).
- [61] E, Nguyen, JG, Grajales-Reyes, RW, Gereau, SE, Ross. Cell type-specific dissection of sensory pathways involved in descending modulation - PubMed. *Trends in neurosciences* 2023 Jul;46(7).
- [62] Ossipov, Michael H, Morimura, Kozo, Porreca, Frank. Descending pain modulation and chronification of pain. *Current opinion in supportive and palliative care* 2014 Jun;8(2).
- [63] François, Amaury, Low, Sarah A, Sypek, Elizabeth I, Christensen, Amelia J, Sotoudeh, Chaudy, Beier, Kevin T, Ramakrishnan, Charu, Ritola, Kimberly D, Sharif-Naeini, Reza, Deisseroth, Karl, Delp, Scott L, Malenka, Robert C, Luo, Liqun, Hantman, Adam W, Scherrer, Grégory. A Brainstem-Spinal Cord Inhibitory Circuit for Mechanical Pain Modulation by GABA and Enkephalins. *Neuron* 2017 Feb 2;93(4).
- [64] Peng, Bingxue, Jiao, Yingfu, Zhang, Yunchun, Li, Shian, Chen, Sihan, Xu, Saihong, Gao, Po, Fan, Yinghui, Yu, Weifeng. *Frontiers | Bulbospinal nociceptive ON and OFF cells related neural circuits and transmitters. Frontiers in Pharmacology* 2023/04/20;14.
- [65] Cao, Bo, Xu, Qixuan, Shi, Yajiao, Zhao, Ruiyang, Li, Hanghang, Zheng, Jie, Liu, Fengyu, Wan, You, Wei, Bo, Cao, Bo, Xu, Qixuan, Shi, Yajiao, Zhao, Ruiyang, Li, Hanghang, Zheng, Jie, Liu, Fengyu, Wan, You, Wei, Bo. Pathology of pain and its implications for therapeutic interventions. *Signal Transduction and Targeted Therapy* 2024 9:1 2024-06-08;9(1).
- [66] Vadhel, Agrataben, Bashir, Sabreen, Mir, Ashiq Hussain, Girdhar, Madhuri, Kumar, Deepak, Kumar, Anil, Mohan, Aradhana, Malik, Tabarak, Mohan, Anand. Opium alkaloids, biosynthesis, pharmacology and association with cancer occurrence. *Open Biology* 2023 May 3;13(5).
- [67] Reeves, K. C., Shah, N., Munoz, B., Atwood, B. K. Opioid Receptor-Mediated Regulation of Neurotransmission in the Brain. *Front Mol Neurosci* 2022;15:919773.
- [68] Stein, Christoph. Opioids, sensory systems and chronic pain. *European Journal of Pharmacology* 2013/09/15;716(1-3).
- [69] T, Trang, R, Al-Hasani, D, Salvemini, MW, Salter, H, Gutstein, CM, Cahill. Pain and Poppies: The Good, the Bad, and the Ugly of Opioid Analgesics - PubMed. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 10/14/2015;35(41).
- [70] Endres-Becker, Jeannette, Heppenstall, Paul A., Mousa, Shaaban A., Labuz, Dominika, Oksche, Alexander, Schäfer, Michael, Stein, Christoph, Zöllner, Christian.  $\mu$ -Opioid Receptor Activation Modulates Transient Receptor Potential Vanilloid 1 (TRPV1) Currents in Sensory Neurons in A Model of Inflammatory Pain. *Molecular Pharmacology* 2007/01/01;71(1).

- [71] Malafoglia, Valentina, Ilari, Sara, Vitiello, Laura, Tenti, Michael, Balzani, Eleonora, Muscoli, Carolina, Raffaelli, William, Bonci, Antonello. The Interplay between Chronic Pain, Opioids, and the Immune System. *The Neuroscientist* 2021 Jul 16;28(6).
- [72] Porreca, Frank, Ossipov, Michael H. Nausea and Vomiting Side Effects with Opioid Analgesics during Treatment of Chronic Pain: Mechanisms, Implications, and Management Options. *Pain Medicine* 2009/05/01;10(4).
- [73] L, Shaiova. The management of opioid-related sedation - PubMed. *Current pain and headache reports* 2005 Aug;9(4).
- [74] Kosten, Thomas R, George, Tony P. The Neurobiology of Opioid Dependence: Implications for Treatment. *Science & Practice Perspectives* 2002 Jul;1(1).
- [75] NIDA. Drug Overdose Deaths: Facts and Figures. 2024.
- [76] C, Florence, F, Luo, K, Rice. The economic burden of opioid use disorder and fatal opioid overdose in the United States, 2017 - PubMed. *Drug and alcohol dependence* 01/01/2021;218.
- [77] Luttrell, Louis M., Lefkowitz, Robert J. The role of  $\beta$ -arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of Cell Science* 2002/02/01;115(3).
- [78] Di Chiara, G., Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A* 1988;85(14):5274-5278.
- [79] MERRER, JULIE LE, BECKER, JÉRÔME A J, BEFORT, KATIA, KIEFFER, BRIGITTE L. Reward Processing by the Opioid System in the Brain. *Physiological reviews* 2009 Oct;89(4).
- [80] Woodhams, Stephen G., Sagar, Devi Rani, Burston, James J., Chapman, Victoria. The Role of the Endocannabinoid System in Pain. *Pain Control* 2015.
- [81] Hasan, Khaled M. Cannabis Unveiled: An Exploration of Marijuana's History, Active Compounds, Effects, Benefits, and Risks on Human Health. *Substance Abuse: Research and Treatment* 2023 Jun 20;17.
- [82] Bridgeman, M. B., Abazia, D. T. Medicinal Cannabis: History, Pharmacology, And Implications for the Acute Care Setting. *P T* 2017;42(3):180-188.
- [83] Moore, Catherine F, Weerts, Elise M. Cannabinoid tetrad effects of oral  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) in male and female rats: sex, dose-effects and time course evaluations. *Psychopharmacology* 2021 Oct 15;239(5).
- [84] Laprairie, R B, Bagher, A M, Kelly, M E M, Denovan-Wright, E M. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British Journal of Pharmacology* 2015 Oct 13;172(20).
- [85] Ryan, Jennie E, McCabe, Sean Esteban, Boyd, Carol J. Medicinal Cannabis: Policy, Patients, and Providers. *Policy, politics & nursing practice* 2021 Feb 10;22(2).
- [86] SH, Gage, M, Hickman, S, Zammit. Association Between Cannabis and Psychosis: Epidemiologic Evidence - PubMed. *Biological psychiatry* 04/01/2016;79(7).
- [87] National Academies of Sciences, Engineering, and Medicine, National Academies of Sciences, Engineering, and Medicine. The Health Effects of Cannabis and Cannabinoids: The Current State of Evidence and Recommendations for Research. 2017/01/12.

- [88] Leung, Janni, Chan, Gary, Stjepanović, Daniel, Chung, Jack Yiu Chak, Hall, Wayne, Hammond, David. Prevalence and self-reported reasons of cannabis use for medical purposes in USA and Canada. *Psychopharmacology* 2022 Jan 12;239(5).
- [89] V, Katsidoni, A, Kastellakis, G, Panagis. Biphasic effects of  $\Delta^9$ -tetrahydrocannabinol on brain stimulation reward and motor activity - PubMed. *The international journal of neuropsychopharmacology* 2013 Nov;16(10).
- [90] Bloomfield, Michael AP, Ashok, Abhishekh H, Volkow, Nora D, Howes, Oliver D. The effects of  $\Delta^9$ -tetrahydrocannabinol on the dopamine system. *Nature* 2016 Nov 17;539(7629).
- [91] Liktor-Busa, E., Keresztes, A., LaVigne, J., Streicher, J. M., Largent-Milnes, T. M. Analgesic Potential of Terpenes Derived from *Cannabis sativa*. *Pharmacol Rev* 2021;73(4):98-126.
- [92] LaVigne, J. E., Hecksel, R., Keresztes, A., Streicher, J. M. *Cannabis sativa* terpenes are cannabimimetic and selectively enhance cannabinoid activity. *Sci Rep* 2021;11(1):8232.
- [93] Robinson, Richard. Selective Reduction of THC's Unwanted Effects through Serotonin Receptor Inhibition. *PLoS Biology* 2015 Jul 9;13(7).
- [94] Z, Justinova, P, Mascia, HQ, Wu, ME, Secci, GH, Redhi, LV, Panlilio, M, Scherma, C, Barnes, A, Parashos, T, Zara, W, Fratta, M, Solinas, M, Pistis, J, Bergman, BD, Kangas, S, Ferré, G, Tanda, R, Schwarcz, SR, Goldberg. Reducing cannabinoid abuse and preventing relapse by enhancing endogenous brain levels of kynurenic acid - PubMed. *Nature neuroscience* 2013 Nov;16(11).
- [95] AM, Schwarz, D, Kobeci, JA, Mancuso, V, Moreno-Rodríguez, C, Seekins, T, Bui, A, Welborn, J, Carr, JM, Streicher. Select Minor Cannabinoids from *Cannabis sativa* Are Cannabimimetic and Antinociceptive in a Mouse Model of Chronic Neuropathic Pain - PubMed. *The Journal of pharmacology and experimental therapeutics* 10/18/2024;391(2).
- [96] Berger, Miles, Gray, John A, Roth, Bryan L. The Expanded Biology of Serotonin. *Annual review of medicine* 2009;60(1).
- [97] WK, Kroeze, K, Kristiansen, BL, Roth. Molecular biology of serotonin receptors structure and function at the molecular level - PubMed. *Current topics in medicinal chemistry* 2002 Jun;2(6).
- [98] Marks, David M, Shah, Manan J, Patkar, Ashwin A, Masand, Prakash S, Park, Geun-Young, Pae, Chi-Un. Serotonin-Norepinephrine Reuptake Inhibitors for Pain Control: Premise and Promise. *Current Neuropharmacology* 2009 Dec;7(4).
- [99] Patetsos, Elias, Horjales-Araujo, Emilia. Treating Chronic Pain with SSRIs: What Do We Know? *Pain Research & Management* 2016 Jul 3;2016.
- [100] Sindrup, Søren H., Otto, Marit, Finnerup, Nanna B., Jensen, Troels S. Antidepressants in the Treatment of Neuropathic Pain. *Basic & Clinical Pharmacology & Toxicology* 2005/06/01;96(6).
- [101] Ganzberg, Steven. Pain Management Part II: Pharmacologic Management of Chronic Orofacial Pain. *Anesthesia Progress* 2010 Fall;57(3).
- [102] CA, Pettengill, L, Reisner-Keller. The use of tricyclic antidepressants for the control of chronic orofacial pain - PubMed. *Cranio : the journal of craniomandibular practice* 1997 Jan;15(1).
- [103] JG, Ochoa, WA, Kilgo. The Role of Benzodiazepines in the Treatment of Epilepsy - PubMed. *Current treatment options in neurology* 2016 Apr;18(4).

- [104] Kienitz, Ricardo, Kay, Lara, Beuchat, Isabelle, Gelhard, Sarah, Brauchitsch, Sophie von, Mann, Catrin, Lucaciu, Alexandra, Schäfer, Jan-Hendrik, Siebenbrodt, Kai, Zöllner, Johann-Philipp, Schubert-Bast, Susanne, Rosenow, Felix, Strzelczyk, Adam, Willems, Laurent M. Benzodiazepines in the Management of Seizures and Status Epilepticus: A Review of Routes of Delivery, Pharmacokinetics, Efficacy, and Tolerability. *CNS Drugs* 2022 Aug 16;36(9).
- [105] Chincholkar, Mahindra. Gabapentinoids: pharmacokinetics, pharmacodynamics and considerations for clinical practice. *British Journal of Pain* 2020 Mar 13;14(2).
- [106] S, Reddy, RB, Patt. The benzodiazepines as adjuvant analgesics - PubMed. *Journal of pain and symptom management* 1994 Nov;9(8).
- [107] Hendrich, Jan, Minh, Alexandra Tran Van, Heblich, Fay, Nieto-Rostro, Manuela, Watschinger, Katrin, Striessnig, Jörg, Wratten, Jack, Davies, Anthony, Dolphin, Annette C., Hendrich, Jan, Van Minh, Alexandra Tran, Heblich, Fay, Nieto-Rostro, Manuela, Watschinger, Katrin, Striessnig, Jörg, Wratten, Jack, Davies, Anthony, Dolphin, Annette C. Pharmacological disruption of calcium channel trafficking by the  $\alpha 2\delta$  ligand gabapentin. *Proceedings of the National Academy of Sciences* 2008-3-4;105(9).
- [108] Thomas R. Belliotti, †, †, Thomas Capiris, †, I. Victor Ekhato, †, †, Jack J. Kinsora, †, Mark J. Field, ‡, Thomas G. Heffner, §, Leonard T. Meltzer, †, Jacob B. Schwarz, †, Charles P. Taylor, †, Andrew J. Thorpe, †, Mark G. Vartanian, †, Lawrence D. Wise, †, Ti Zhi-Su, †, Mark L. Weber, † and, David J. Wustrow†, \*. Structure–Activity Relationships of Pregabalin and Analogues That Target the  $\alpha 2\text{-}\delta$  Protein. February 17, 2005.
- [109] Field, Mark J., Cox, Peter J., Stott, Emma, Melrose, Heather, Offord, James, Su, Ti-Zhi, Bramwell, Steve, Corradini, Laura, England, Steven, Winks, Joanna, Kinloch, Ross A., Hendrich, Jan, Dolphin, Annette C., Webb, Tony, Williams, Dic, Field, Mark J., Cox, Peter J., Stott, Emma, Melrose, Heather, Offord, James, Su, Ti-Zhi, Bramwell, Steve, Corradini, Laura, England, Steven, Winks, Joanna, Kinloch, Ross A., Hendrich, Jan, Dolphin, Annette C., Webb, Tony, Williams, Dic. Identification of the  $\alpha 2\text{-}\delta\text{-}1$  subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. *Proceedings of the National Academy of Sciences* 2006-11-14;103(46).
- [110] Li, Chun-Ying, Zhang, Xiu-Lin, Matthews, Elizabeth A., Li, Kang-Wu, Kurwa, Ambereen, Boroujerdi, Amin, Gross, Jimmy, Gold, Michael S., Dickenson, Anthony H., Feng, Guoping, Luo, Z. David. Calcium channel  $\alpha 2\delta 1$  subunit mediates spinal hyperexcitability in pain modulation. *Pain* 2006/11/01;125(1-2).
- [111] Nørregaard, Rikke, Kwon, Tae-Hwan, Frøkiær, Jørgen. Physiology and pathophysiology of cyclooxygenase-2 and prostaglandin E2 in the kidney. *Kidney Research and Clinical Practice* 2015 Nov 12;34(4).
- [112] I, Morita. Distinct functions of COX-1 and COX-2 - PubMed. *Prostaglandins & other lipid mediators* 2002 Aug;68-69.
- [113] Vane, J.R, Botting, R.M. The mechanism of action of aspirin. *Thrombosis Research* 2003/06/15;110(5-6).
- [114] Bushra, Rabia, Aslam, Nousheen. An Overview of Clinical Pharmacology of Ibuprofen. *Oman Medical Journal* 2010 Jul;25(3).

- [115] Ohashi, Nobuko, Kohno, Tatsuro. Frontiers | Analgesic Effect of Acetaminophen: A Review of Known and Novel Mechanisms of Action. *Frontiers in Pharmacology* 2020/11/30;11.
- [116] B, Hinz, O, Cheremina, K, Brune. Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man - PubMed. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2008 Feb;22(2).
- [117] Salis, Zubeyir, Sainsbury, Amanda, Salis, Zubeyir, Sainsbury, Amanda. Association of long-term use of non-steroidal anti-inflammatory drugs with knee osteoarthritis: a prospective multi-cohort study over 4-to-5 years. *Scientific Reports* 2024 14:1 2024-03-19;14(1).
- [118] Marcum, Z. A., Hanlon, J. T. Recognizing the Risks of Chronic Nonsteroidal Anti-Inflammatory Drug Use in Older Adults. *Ann Longterm Care* 2010;18(9):24-27.
- [119] Ricciotti, Emanuela, FitzGerald, Garret A. Prostaglandins and Inflammation. *Arteriosclerosis, thrombosis, and vascular biology* 2011 May;31(5).
- [120] Wallace, John L. Prostaglandins, NSAIDs, and Gastric Mucosal Protection: Why Doesn't the Stomach Digest Itself? *Physiological Reviews* 2008 Oct 01;88(4).
- [121] Hermanns, Henning, Hollmann, Markus W., Stevens, Markus F., Lirk, Philipp, Brandenburger, Timo, Piegeler, Tobias, Werdehausen, Robert. Molecular mechanisms of action of systemic lidocaine in acute and chronic pain: a narrative review. *British Journal of Anaesthesia* 2019/09/01;123(3).
- [122] Hoffman, E Matthew, Watson, James C, Sauver, Jennifer St, Staff, Nathan P, Klein, Christopher J. Association of Long-term Opioid Therapy With Functional Status, Adverse Outcomes, and Mortality Among Patients With Polyneuropathy. *JAMA Neurology* 2017 Jul 10;74(7).
- [123] Shah, Arya, Hoffman, E Matthew, Mauermann, Michelle L, Loprinzi, Charles L, Windebank, Anthony J, Klein, Christopher J, Staff, Nathan P. Incidence and disease burden of chemotherapy-induced peripheral neuropathy in a population-based cohort. *Journal of neurology, neurosurgery, and psychiatry* 2018 Feb 8;89(6).
- [124] HV, Curran, TP, Freeman, C, Mokrysz, DA, Lewis, CJ, Morgan, LH, Parsons. Keep off the grass? Cannabis, cognition and addiction - PubMed. *Nature reviews Neuroscience* 2016 May;17(5).
- [125] PA, Barber, S, Roberts, DA, Spriggs, NE, Anderson. Adverse cardiovascular, cerebrovascular, and peripheral vascular effects of marijuana: what cardiologists need to know - PubMed. *The American journal of cardiology* 03/15/2014;113(6).
- [126] M, Joshi, A, Joshi, T, Bartter. Marijuana and lung diseases - PubMed. *Current opinion in pulmonary medicine* 2014 Mar;20(2).
- [127] Jackson, Jeffrey L, Damm, Tessa W. Barriers to the Use of Tricyclic Antidepressants in the Management of Pain. *Pain Management* 2011-3-1;1(2).
- [128] Ho, Kok Yuen, Gwee, Kok Ann, Cheng, Yew Kuang, Yoon, Kam Hon, Hee, Hwan Tak, Omar, Abdul Razakjr. Nonsteroidal anti-inflammatory drugs in chronic pain: implications of new data for clinical practice. *Journal of Pain Research* 2018 Sep 20;11.
- [129] R, Fredriksson, MC, Lagerström, LG, Lundin, HB, Schiöth. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints - PubMed. *Molecular pharmacology* 2003 Jun;63(6).
- [130] Sriram, Krishna, Insel, Paul A. G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs? *Molecular Pharmacology* 2018 Apr;93(4).

- [131] C, Laschet, N, Dupuis, J, Hanson. The G protein-coupled receptors deorphanization landscape - PubMed. *Biochemical pharmacology* 2018 Jul;153.
- [132] Öz-Arslan, Devrim, Yavuz, Melis, Kan, Beki. Frontiers | Exploring orphan GPCRs in neurodegenerative diseases. *Frontiers in Pharmacology* 2024/06/04;15.
- [133] X, Xiang, X, Wang, Y, Wu, J, Hu, Y, Li, S, Jin, X, Wu. Activation of GPR55 attenuates cognitive impairment, oxidative stress, neuroinflammation, and synaptic dysfunction in a streptozotocin-induced Alzheimer's mouse model - PubMed. *Pharmacology, biochemistry, and behavior* 2022 Mar;214.
- [134] J, Audoy-Rémus, L, Bozoyan, A, Dumas, M, Filali, C, Lecours, S, Lacroix, S, Rivest, ME, Tremblay, L, Vallières. GPR84 deficiency reduces microgliosis, but accelerates dendritic degeneration and cognitive decline in a mouse model of Alzheimer's disease - PubMed. *Brain, behavior, and immunity* 2015 May;46.
- [135] Y, Huang, A, Skwarek-Maruszewska, K, Horré, E, Vandewyer, L, Wolfs, A, Snellinx, T, Saito, E, Radaelli, N, Corthout, J, Colombelli, AC, Lo, L, Van Aerschot, Z, Callaerts-Vegh, D, Trabzuni, K, Bossers, J, Verhaagen, M, Ryten, S, Munck, R, D'Hooge, DF, Swaab, J, Hardy, TC, Saïdo, B, De Strooper, A, Thathiah. Loss of GPR3 reduces the amyloid plaque burden and improves memory in Alzheimer's disease mouse models - PubMed. *Science translational medicine* 10/14/2015;7(309).
- [136] W, Chen, H, Ji, L, Li, C, Xu, T, Zou, W, Cui, S, Xu, X, Zhou, S, Duan, Q, Wang. Significant association between GPR50 hypomethylation and AD in males - PubMed. *Molecular medicine reports* 2019 Aug;20(2).
- [137] Matsumoto, Mitsuyuki, Straub, Richard E, Marenco, Stefano, Nicodemus, Kristin K, Matsumoto, Shun-ichiro, Fujikawa, Akihiko, Miyoshi, Sosuke, Shobo, Miwako, Takahashi, Shinji, Yarimizu, Junko, Yuri, Masatoshi, Hiramoto, Masashi, Morita, Shuji, Yokota, Hiroyuki, Sasayama, Takeshi, Terai, Kazuhiro, Yoshino, Masayasu, Miyake, Akira, Callicott, Joseph H, Egan, Michael F, Meyer-Lindenberg, Andreas, Kempf, Lucas, Honea, Robyn, Vakkalanka, Radha Krishna, Takasaki, Jun, Kamohara, Masazumi, Soga, Takatoshi, Hiyama, Hideki, Ishii, Hiroyuki, Matsuo, Ayako, Nishimura, Shintaro, Matsuoka, Nobuya, Kobori, Masato, Matsushime, Hitoshi, Katoh, Masao, Furuichi, Kiyoshi, Weinberger, Daniel R. The evolutionarily conserved G protein-coupled receptor SREB2/GPR85 influences brain size, behavior, and vulnerability to schizophrenia. *P Natl Acad Sci USA* 2008 Apr 14;105(16).
- [138] Y, Imai, M, Soda, H, Inoue, N, Hattori, Y, Mizuno, R, Takahashi. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin - PubMed. *Cell* 06/29/2001;105(7).
- [139] SW, Saliba, H, Jauch, B, Gargouri, A, Keil, T, Hurrle, N, Volz, F, Mohr, M, van der Stelt, S, Bräse, BL, Fiebich. Anti-neuroinflammatory effects of GPR55 antagonists in LPS-activated primary microglial cells - PubMed. *Journal of neuroinflammation* 11/19/2018;15(1).
- [140] D, Medina-Vera, H, Zhao, E, Bereczki, C, Rosell-Valle, M, Shimozawa, G, Chen, FR, de Fonseca, P, Nilsson, S, Tambaro. The Expression of the Endocannabinoid Receptors CB2 and GPR55 Is Highly Increased during the Progression of Alzheimer's Disease in AppNL-G-F Knock-In Mice - PubMed. *Biology* 05/31/2023;12(6).

- [141] He, Yi, Shen, Hui, Bi, Guo-Hua, Zhang, Hai-Ying, Soler-Cedeño, Omar, Alton, Hannah, Yang, Yihong, Xi, Zheng-Xiong, He, Yi, Shen, Hui, Bi, Guo-Hua, Zhang, Hai-Ying, Soler-Cedeño, Omar, Alton, Hannah, Yang, Yihong, Xi, Zheng-Xiong. GPR55 is expressed in glutamate neurons and functionally modulates drug taking and seeking in rats and mice. *Translational Psychiatry* 2024 14:1 2024-02-19;14(1).
- [142] F, Patricio, E, Morales Dávila, A, Patricio-Martínez, N, Arana Del Carmen, I, Martínez, J, Aguilera, JM, Perez-Aguilar, ID, Limón. Intrapallidal injection of cannabidiol or a selective GPR55 antagonist decreases motor asymmetry and improves fine motor skills in hemiparkinsonian rats - PubMed. *Frontiers in pharmacology* 09/02/2022;13.
- [143] Ehrlich, Aliza T., Maroteaux, Grégoire, Robe, Anne, Venteo, Lydie, Nasseef, Md Taufiq, van Kempen, Leon C., Mechawar, Naguib, Turecki, Gustavo, Darcq, Emmanuel, Kieffer, Brigitte L. Expression map of 78 brain-expressed mouse orphan GPCRs provides a translational resource for neuropsychiatric research. *Communications Biology*, Vol. 1, 2018.
- [144] Kakarala, Kavita Kumari, Jamil, Kaiser. Sequence-structure based phylogeny of GPCR Class A Rhodopsin receptors. *Molecular Phylogenetics and Evolution*, Vol. 74, 2014.
- [145] Tavares-Ferreira, D., Shiers, S., Ray, P. R., Wangzhou, A., Jeevakumar, V., Sankaranarayanan, I., Cervantes, A. M., Reese, J. C., Chamesian, A., Copits, B. A., Dougherty, P. M., Gereau, R. W. th, Burton, M. D., Dussor, G., Price, T. J. Spatial transcriptomics of dorsal root ganglia identifies molecular signatures of human nociceptors. *Sci Transl Med* 2022;14(632):eabj8186.
- [146] Im, Dong-Soon. Discovery of new G protein-coupled receptors for lipid mediators. *Journal of Lipid Research* 2004/03/01;45(3).
- [147] Afrose, Leela, Gomes, Ivone, Devi, Lakshmi A., Bobeck, Erin N. The Neuropeptide Receptor System, BigLEN-GPR171, Interacts with the Opioid System to Relieve Pain. *The FASEB Journal*, Vol. 32, 2018.
- [148] McDermott, Max V., Afrose, Leela, Gomes, Ivone, Devi, Lakshmi A., Bobeck, Erin N. Opioid-induced signaling and antinociception are modulated by the recently deorphanized receptor, GPR171S. *Journal of Pharmacology and Experimental Therapeutics*, Vol. 371, 2019.
- [149] Edwards, Talyor M., Ram, Akila, McCarty, Ashley, Bobeck, Erin N. Role of novel receptor GPR171 in chemotherapy-induced neuropathic pain. *The FASEB Journal*, Vol. 34, 2020.
- [150] McDermott, Max, Bobeck, Erin. GPR171 Agonist, MS15203, Effectively Enhances Morphine Antinociception Without Modulating Detrimental Side Effects. *The FASEB Journal*, Vol. 35, 2021.
- [151] Laboute, Thibaut, Gandía, Jorge, Pellissier, Lucie P., Corde, Yannick, Rebeillard, Florian, Gallo, Maria, Gauthier, Christophe, Léauté, Audrey, Diaz, Jorge, Poupon, Anne, Kieffer, Brigitte L., Le Merrer, Julie, Becker, Jérôme Aj. The orphan receptor GPR88 blunts the signaling of opioid receptors and multiple striatal GPCRs. *eLife* 2020;9.
- [152] RM, Schafer, LA, Giancotti, DJ, Davis, IG, Larrea, SA, Farr, D, Salvemini. Behavioral characterization of G-protein-coupled receptor 160 knockout mice - PubMed. *Pain* 06/01/2024;165(6).

- [153] Y, Kim, C, Kim, H, Lee, M, Kim, H, Zheng, JY, Lim, HI, Yun, M, Jeon, J, Choi, SW, Hwang. Gpr83 Tunes Nociceptor Function, Controlling Pain - PubMed. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 2023 Jan;20(1).
- [154] K, Nakamoto, T, Nishinaka, K, Matsumoto, F, Kasuya, M, Mankura, Y, Koyama, S, Tokuyama. Involvement of the long-chain fatty acid receptor GPR40 as a novel pain regulatory system - PubMed. *Brain research* 01/13/2012;1432.
- [155] Xie, Y. K., Luo, H., Zhang, S. X., Chen, X. Y., Guo, R., Qiu, X. Y., Liu, S., Wu, H., Chen, W. B., Zhen, X. H., Ma, Q., Tian, J. L., Li, S., Chen, X., Han, Q., Duan, S., Shen, C., Yang, F., Xu, Z. Z. GPR177 in A-fiber sensory neurons drives diabetic neuropathic pain via WNT-mediated TRPV1 activation. *Sci Transl Med* 2022;14(639):eabh2557.
- [156] Kern, Katharina, Schäfer, Stephan M. G., Cohnen, Jennifer, Pierre, Sandra, Osthues, Tabea, Tarighi, Neda, Hohmann, Stefan, Ferreiros, Nerea, Brüne, Bernhard, Weigert, Andreas, Geisslinger, Gerd, Sisignano, Marco, Scholich, Klaus. *Frontiers | The G2A Receptor Controls Polarization of Macrophage by Determining Their Localization Within the Inflamed Tissue. Frontiers in Immunology* 2018/10/01;9.
- [157] Yong, R. Jason, Mullins, Peter M., Bhattacharyya, Neil. Prevalence of chronic pain among adults in the United States. *Pain*, Vol. 163, 2022.
- [158] Barke, A., Korwisi, B., Jakob, R., Konstanjsek, N., Rief, W., Treede, R. D. Classification of chronic pain for the International Classification of Diseases (ICD-11): results of the 2017 international World Health Organization field testing. *Pain* 2022;163(2):e310-e318.
- [159] Coussens, N. P., Sittampalam, G. S., Jonson, S. G., Hall, M. D., Gorby, H. E., Tamiz, A. P., McManus, O. B., Felder, C. C., Rasmussen, K. The Opioid Crisis and the Future of Addiction and Pain Therapeutics. *J Pharmacol Exp Ther* 2019;371(2):396-408.
- [160] Yang, D., Zhou, Q., Labroska, V., Qin, S., Darbalaei, S., Wu, Y., Yuliantie, E., Xie, L., Tao, H., Cheng, J., Liu, Q., Zhao, S., Shui, W., Jiang, Y., Wang, M. W. G protein-coupled receptors: structure- and function-based drug discovery. *Signal Transduct Target Ther* 2021;6(1):7.
- [161] AS, Hauser, MM, Attwood, M, Rask-Andersen, HB, Schiöth, DE, Gloriam. Trends in GPCR drug discovery: new agents, targets and indications - PubMed. *Nature reviews Drug discovery* 2017 Dec;16(12).
- [162] Nourbakhsh, Fahimeh, Atabaki, Rabi, Roohbakhsh, Ali. The role of orphan G protein-coupled receptors in the modulation of pain: A review. *Life Sciences*, Vol. 212, 2018. pp. 59-69.
- [163] Safran, Marilyn, Rosen, Naomi, Twik, Michal, BarShir, Ruth, Stein, Tsippi Iny, Dahary, Dvir, Fishilevich, Simon, Lancet, Doron. *The GeneCards Suite. Practical Guide to Life Science Databases*, 2021.
- [164] Niedernberg, Anke, Tunaru, Sorin, Blaukat, Andree, Ardati, Ali, Kostenis, Evi. Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63. *Cellular Signalling*, Vol. 15, 2003.
- [165] Squillace, Silvia, Spiegel, Sarah, Salvemini, Daniela. Targeting the Sphingosine-1-Phosphate Axis for Developing Non-narcotic Pain Therapeutics. *Trends in Pharmacological Sciences*, Vol. 41, 2020.

- [166] Derwińska, K., Bernaciak, J., Wiśniowiecka-Kowalnik, B., Obersztyn, E., Bocian, E., Stankiewicz, P. Autistic features with speech delay in a girl with an ~1.5-Mb deletion in 6q16.1, including GPR63 and FUT9. *Clinical Genetics*, Vol. 75, 2009.
- [167] Snedeker, John, Gibbons, William J., Paulding, David F., Abdelhamed, Zakia, Prows, Daniel R., Stottmann, Rolf W. Gpr63 is a modifier of microcephaly in Ttc21b mouse mutants. *PLoS Genetics*, Vol. 15, 2019.
- [168] Mitra, I., Lavillaureix, A., Yeh, E., Traglia, M., Tsang, K., Bearden, C. E., Rauen, K. A., Weiss, L. A. Reverse Pathway Genetic Approach Identifies Epistasis in Autism Spectrum Disorders. *PLoS Genet* 2017;13(1):e1006516.
- [169] Kohno, Masashi, Hasegawa, Hitoshi, Inoue, Atsushi, Muraoka, Masatake, Miyazaki, Tatsuhiko, Oka, Keizo, Yasukawa, Masaki. Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochemical and Biophysical Research Communications*, Vol. 347, 2006.
- [170] Vuong, Leeza A.Q., Mitchell, Vanessa A., Vaughan, Christopher W. Actions of N-arachidonyl-glycine in a rat neuropathic pain model. *Neuropharmacology*, Vol. 54, 2008.
- [171] Nees, F., Loffler, M., Usai, K., Flor, H. Hypothalamic-pituitary-adrenal axis feedback sensitivity in different states of back pain. *Psychoneuroendocrinology* 2019;101:60-66.
- [172] Sreedharan, Smitha, Almén, Markus S., Carlini, Valeria P., Haitina, Tatjana, Stephansson, Olga, Sommer, Wolfgang H., Heilig, Markus, De Barioglio, Susana R., Fredriksson, Robert, Schiöth, Helgi B. The G protein coupled receptor Gpr153 shares common evolutionary origin with Gpr162 and is highly expressed in central regions including the thalamus, cerebellum and the arcuate nucleus. *FEBS Journal*, Vol. 278, 2011.
- [173] K, Pandey, O, Kereilwe, H, Kadokawa. Heifers express G-protein coupled receptor 153 in anterior pituitary gonadotrophs in stage-dependent manner - PubMed. *Animal science journal = Nihon chikusan Gakkaiho* 2018 Jan;89(1).
- [174] PANDEY, Kiran, MIZUKAMI, Yoichi, WATANABE, Kenji, SAKAGUTI, Syuiti, KADOKAWA, Hiroya. Deep sequencing of the transcriptome in the anterior pituitary of heifers before and after ovulation. *The Journal of Veterinary Medical Science* 2017 Apr 23;79(6).
- [175] KADOKAWA, Hiroya. Discovery of new receptors regulating luteinizing hormone and follicle-stimulating hormone secretion by bovine gonadotrophs to explore a new paradigm for mechanisms regulating reproduction. *The Journal of Reproduction and Development* 2020 Apr 6;66(4).
- [176] A, Ruiz-Hernández, S, Cabrera-Becerra, G, Vera-Juárez, E, Hong, H, Fengyang, J, Arauz, S, Villafaña. Diabetic nephropathy produces alterations in the tissue expression profile of the orphan receptors GPR149, GPR153, GPR176, TAAR3, TAAR5 and TAAR9 in Wistar rats - PubMed. *Nucleosides, nucleotides & nucleic acids* 2020;39(8).
- [177] Deuis, Jennifer R, Dvorakova, Lucie S, Vetter, Irina. Methods Used to Evaluate Pain Behaviors in Rodents. *Frontiers in Molecular Neuroscience* 2017 Sep 6;10.
- [178] Cowie, Ashley M, Stucky, Cheryl L. A Mouse Model of Postoperative Pain. *Bio-protocol* 2019 Jan 20;9(2).
- [179] Bankhead, Peter, Loughrey, Maurice B., Fernández, José A., Dombrowski, Yvonne, McArt, Darragh G., Dunne, Philip D., McQuaid, Stephen, Gray, Ronan T., Murray, Liam J., Coleman, Helen G., James, Jacqueline A., Salto-Tellez, Manuel, Hamilton, Peter W.,

- Bankhead, Peter, Loughrey, Maurice B., Fernández, José A., Dombrowski, Yvonne, McArt, Darragh G., Dunne, Philip D., McQuaid, Stephen, Gray, Ronan T., Murray, Liam J., Coleman, Helen G., James, Jacqueline A., Salto-Tellez, Manuel, Hamilton, Peter W. QuPath: Open source software for digital pathology image analysis. *Scientific Reports* 2017 7:1 2017-12-04;7(1).
- [180] Lei, Wei, Vekariya, Rakesh H, Ananthan, Subramaniam, Streicher, John M. A Novel Mu-Delta Opioid Agonist Demonstrates Enhanced Efficacy with Reduced Tolerance and Dependence in Mouse Neuropathic Pain Models. *The journal of pain : official journal of the American Pain Society* 2019 Jun 12;21(1-2).
- [181] Weaver, Beth A. How Taxol/paclitaxel kills cancer cells. *Molecular Biology of the Cell* 2014 Sep 15;25(18).
- [182] TM, Mekhail, M, Markman. Paclitaxel in cancer therapy - PubMed. *Expert opinion on pharmacotherapy* 2002 Jun;3(6).
- [183] Glare, Paul, Aubrey, Karin R, Myles, Paul S. Transition from acute to chronic pain after surgery. *The Lancet* 2019/04/13;393(10180).
- [184] Zhu, Kun, Wang, Lin, Liao, TingTing, Li, Wen, Zhou, Jing, You, Yaodong, Shi, Jianyou. Progress in the development of TRPV1 small-molecule antagonists: Novel Strategies for pain management. *European Journal of Medicinal Chemistry* 2023/12/05;261.
- [185] Hole, Kjell, Tjølsen, Arne. Tail Flick Test. 2007.
- [186] Jirkof, Paulin, Cesarovic, Nikola, Rettich, Andreas, Nicholls, Flora, Seifert, Burkhardt, Arras, Margarete. Burrowing Behavior as an Indicator of Post-Laparotomy Pain in Mice. *Frontiers in Behavioral Neuroscience* 2010 Oct 12;4.
- [187] DJ, Langford, AL, Bailey, ML, Chanda, SE, Clarke, TE, Drummond, S, Echols, S, Glick, J, Ingrao, T, Klassen-Ross, ML, Lacroix-Fralish, L, Matsumiya, RE, Sorge, SG, Sotocinal, JM, Tabaka, D, Wong, AM, van den Maagdenberg, MD, Ferrari, KD, Craig, JS, Mogil. Coding of facial expressions of pain in the laboratory mouse - PubMed. *Nature methods* 2010 Jun;7(6).
- [188] F, Gaston-Johansson, M, Albert, E, Fagan, L, Zimmerman. Similarities in pain descriptions of four different ethnic-culture groups - PubMed. *Journal of pain and symptom management* 1990 Apr;5(2).
- [189] SE, Bove, SL, Calcaterra, RM, Brooker, CM, Huber, RE, Guzman, PL, Juneau, DJ, Schrier, KS, Kilgore. Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis - PubMed. *Osteoarthritis and cartilage* 2003 Nov;11(11).
- [190] Xu, Jing, Yan, Zihan, Bang, Sangsu, Velmeshev, Dmitry, Ji, Ru-Rong. GPR37L1 identifies spinal cord astrocytes and protects neuropathic pain after nerve injury. *Neuron* 2025/02/13.
- [191] Chiang, C.-Y., Sessle, B. J., Dostrovsky, J. O., Chiang, C.-Y., Sessle, B. J., Dostrovsky, J. O. Role of Astrocytes in Pain. *Neurochemical Research* 2012 37:11 2012-05-26;37(11).
- [192] Tsuda, Makoto, Kohro, Yuta, Yano, Takayuki, Tsujikawa, Tomoko, Kitano, Junko, Tozaki-Saitoh, Hidetoshi, Koyanagi, Satoru, Ohdo, Shigehiro, Ji, Ru-Rong, Salter, Michael W., Inoue, Kazuhide. JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. *Brain* 2011/04/01;134(4).
- [193] Ji, Ru-Rong, Suter, Marc R. p38 MAPK, microglial signaling, and neuropathic pain. *Molecular Pain* 2007 Nov 1;3(1).

- [194] Ji, R. R., Berta, T., Nedergaard, M. Glia and pain: is chronic pain a gliopathy? *Pain* 2013;154 Suppl 1(0 1):S10-S28.
- [195] Eidson, Lori N, Murphy, Anne Z. Blockade of Toll-Like Receptor 4 Attenuates Morphine Tolerance and Facilitates the Pain Relieving Properties of Morphine. *The Journal of Neuroscience* 2013 Oct 2;33(40).
- [196] Eidson, Lori N, Inoue, Kiyoshi, Young, Larry J, Tansey, Malu G, Murphy, Anne Z. Toll-like Receptor 4 Mediates Morphine-Induced Neuroinflammation and Tolerance via Soluble Tumor Necrosis Factor Signaling. *Neuropsychopharmacology* 2016 Aug 17;42(3).
- [197] Liu, Xin, Bae, Chilman, Liu, Bolong, Zhang, Yong-Mei, Zhou, Xiangfu, Zhang, Donghang, Zhou, Cheng, DiBua, Adriana, Schutz, Livia, Kaczocha, Martin, Puopolo, Michelino, Yamaguchi, Terry P, Chung, Jin Mo, Tang, Shao-Jun. Development of opioid-induced hyperalgesia depends on reactive astrocytes controlled by Wnt5a signaling. *Molecular psychiatry* 2022 Oct 6;28(2).
- [198] A, Bessis, C, Béchade, D, Bernard, A, Roumier. Microglial control of neuronal death and synaptic properties - PubMed. *Glia* 2007 Feb;55(3).
- [199] I, Ferrer, E, Bernet, E, Soriano, T, del Rio, M, Fonseca. Naturally occurring cell death in the cerebral cortex of the rat and removal of dead cells by transitory phagocytes - PubMed. *Neuroscience* 1990;39(2).
- [200] CT, Ekdahl, Z, Kokaia, O, Lindvall. Brain inflammation and adult neurogenesis: the dual role of microglia - PubMed. *Neuroscience* 02/06/2009;158(3).
- [201] Piano, Iliaria, Votta, Arianna, Colucci, Patrizia, Corsi, Francesca, Vitolo, Sara, Cerri, Chiara, Puppi, Dario, Lai, Michele, Maya-Vetencourt, José Fernando, Leigheb, Massimiliano, Gabellini, Chiara, Ferraro, Elisabetta, Piano, Iliaria, Votta, Arianna, Colucci, Patrizia, Corsi, Francesca, Vitolo, Sara, Cerri, Chiara, Puppi, Dario, Lai, Michele, Maya-Vetencourt, José Fernando, Leigheb, Massimiliano, Gabellini, Chiara, Ferraro, Elisabetta. Anti-inflammatory reprogramming of microglia cells by metabolic modulators to counteract neurodegeneration; a new role for Ranolazine. *Scientific Reports* 2023 13:1 2023-11-17;13(1).
- [202] Fu, Ruying, Shen, Qingyu, Xu, Pengfei, Luo, Jin Jun, Tang, Yamei. Phagocytosis of Microglia in the Central Nervous System Diseases. *Molecular Neurobiology* 2014 Jan 7;49(3).
- [203] Smith, Joshua A., Das, Arabinda, Ray, Swapan K., Banik, Naren L. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin* 2012/01/04;87(1).
- [204] Marty-Lombardi, Sebastián, Lu, Shiyong, Ambroziak, Wojciech, Schrenk-Siemens, Katrin, Wang, Jialin, DePaoli-Roach, Anna A., Hagenston, Anna M., Wende, Hagen, Tappe-Theodor, Anke, Simonetti, Manuela, Bading, Hilmar, Okun, Jürgen G., Kuner, Rohini, Fleming, Thomas, Siemens, Jan, Marty-Lombardi, Sebastián, Lu, Shiyong, Ambroziak, Wojciech, Schrenk-Siemens, Katrin, Wang, Jialin, DePaoli-Roach, Anna A., Hagenston, Anna M., Wende, Hagen, Tappe-Theodor, Anke, Simonetti, Manuela, Bading, Hilmar, Okun, Jürgen G., Kuner, Rohini, Fleming, Thomas, Siemens, Jan. Neuron–astrocyte metabolic coupling facilitates spinal plasticity and maintenance of inflammatory pain. *Nature Metabolism* 2024 6:3 2024-03-05;6(3).

- [205] Beard, Elidie, Lengacher, Sylvain, Dias, Sara, Magistretti, Pierre J, Finsterwald, Charles. Astrocytes as Key Regulators of Brain Energy Metabolism: New Therapeutic Perspectives. *Frontiers in Physiology* 2022 Jan 11;12.
- [206] Bonvento, Gilles, Bolaños, Juan P. Astrocyte-neuron metabolic cooperation shapes brain activity. *Cell Metabolism* 2021/08/03;33(8).
- [207] Chung, Won-Suk, Allen, Nicola J, Eroglu, Cagla. Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harbor Perspectives in Biology* 2015 Sep;7(9).
- [208] Kerstetter, Amber E, Miller, Robert H. Isolation and Culture of Spinal Cord Astrocytes. *Methods in molecular biology (Clifton, NJ)* 2012;814.
- [209] HR, Parri, TM, Gould, V, Crunelli. Spontaneous astrocytic Ca<sup>2+</sup> oscillations in situ drive NMDAR-mediated neuronal excitation - PubMed. *Nature neuroscience* 2001 Aug;4(8).
- [210] Chang, Jie, Qian, Zhanyang, Wang, Binyu, Cao, Jiang, Zhang, Sheng, Jiang, Fan, Kong, Renyi, Yu, Xiao, Cao, Xiaojian, Yang, Lei, Chen, Hongtao, Chang, Jie, Qian, Zhanyang, Wang, Binyu, Cao, Jiang, Zhang, Sheng, Jiang, Fan, Kong, Renyi, Yu, Xiao, Cao, Xiaojian, Yang, Lei, Chen, Hongtao. Transplantation of A2 type astrocytes promotes neural repair and remyelination after spinal cord injury. *Cell Communication and Signaling* 2023 21:1 2023-02-16;21(1).
- [211] F, Giovannoni, FJ, Quintana. The Role of Astrocytes in CNS Inflammation - PubMed. *Trends in immunology* 2020 Sep;41(9).
- [212] Ruiz-Sauri, Amparo, Orduña-Valls, Jorge M, Blasco-Serra, Arantxa, Tornero-Tornero, Carlos, Cedeño, David L, Bejarano-Quisoboni, Daniel, Valverde-Navarro, Alfonso A, Benyamin, Ramsin, Vallejo, Ricardo. Glia to neuron ratio in the posterior aspect of the human spinal cord at thoracic segments relevant to spinal cord stimulation. *Journal of Anatomy* 2019 Jul 26;235(5).
- [213] BAHNEY, JAMI, BARTHELD, CHRISTOPHER S VON. The Cellular Composition and Glia-Neuron Ratio in the Spinal Cord of a Human and a Non-Human Primate: Comparison with other Species and Brain Regions. *Anatomical record (Hoboken, NJ : 2007)* 2017 Dec 1;301(4).
- [214] Lu, Huan-Jun, Gao, Yong-Jing. Astrocytes in Chronic Pain: Cellular and Molecular Mechanisms. *Neuroscience Bulletin* 2022 Nov 14;39(3).
- [215] Haight, Elena S, Forman, Thomas E, Cordonnier, Stephanie A, James, Michelle L, Tawfik, Vivianne L. Microglial modulation as a target for chronic pain: From the bench to the bedside and back. *Anesthesia and analgesia* 2019 Apr;128(4).
- [216] Bruno, Kelly, Woller, Sarah A, Miller, Yury I, Yaksh, Tony L, Wallace, Mark, Beaton, Graham, Chakravarthy, Krishnan. Targeting Toll-like receptor-4 (TLR4) – emerging therapeutic target for persistent pain states. *Pain* 2018 Oct;159(10).
- [217] Jin, Shan-Xue, Zhuang, Zhi-Ye, Woolf, Clifford J, Ji, Ru-Rong. p38 Mitogen-Activated Protein Kinase Is Activated after a Spinal Nerve Ligation in Spinal Cord Microglia and Dorsal Root Ganglion Neurons and Contributes to the Generation of Neuropathic Pain. *The Journal of Neuroscience* 2003 May 15;23(10).
- [218] M, Tsuda, Y, Shigemoto-Mogami, S, Koizumi, A, Mizokoshi, S, Kohsaka, MW, Salter, K, Inoue. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury - PubMed. *Nature* 08/14/2003;424(6950).

- [219] K, Inoue, M, Tsuda. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential - PubMed. *Nature reviews Neuroscience* 2018 Mar;19(3).
- [220] Zhang, Xi-Chun, Kainz, Vanessa, Burstein, Rami, Levy, Dan. Tumor necrosis factor- $\alpha$  induces sensitization of meningeal nociceptors mediated via local COX and p38 MAP kinase actions. *Pain* 2010 Oct 30;152(1).
- [221] Romero-Sandoval, Edgar Alfonso, Horvath, Ryan, Landry, Russell P, DeLeo, Joyce A. Cannabinoid receptor type 2 activation induces a microglial anti-inflammatory phenotype and reduces migration via MKP induction and ERK dephosphorylation. *Molecular Pain* 2009 May 28;5(1).
- [222] McKelvey, Rebecca, Berta, Temugin, Old, Elizabeth, Ji, Ru-Rong, Fitzgerald, Maria. Neuropathic Pain Is Constitutively Suppressed in Early Life by Anti-Inflammatory Neuroimmune Regulation. *The Journal of Neuroscience* 2015 Jan 14;35(2).
- [223] Donovan, Lauren J, Bridges, Caldwell M, Nippert, Amy R, Wang, Meng, Wu, Shaogen, Forman, Thomas E, Haight, Elena S, Huck, Nolan A, Bond, Sabrina F, Jordan, Claire E, Gardner, Aysha M, Nair, Ramesh V, Tawfik, Vivianne L. Repopulated spinal cord microglia exhibit a unique transcriptome and contribute to pain resolution. *Cell reports* 2024 Jan 22;43(2).
- [224] Li, Xinyu, Li, Meng, Tian, Lige, Chen, Jianan, Liu, Ronghan, Ning, Bin. Reactive Astrogliosis: Implications in Spinal Cord Injury Progression and Therapy. *Oxidative Medicine and Cellular Longevity* 2020 Aug 19;2020.
- [225] Liddelow, Shane A, Guttenplan, Kevin A, Clarke, Laura E, Bennett, Frederick C, Bohlen, Christopher J, Schirmer, Lucas, Bennett, Mariko L, Münch, Alexandra E, Chung, Won-Suk, Peterson, Todd C, Wilton, Daniel K, Frouin, Arnaud, Napier, Brooke A, Panicker, Nikhil, Kumar, Manoj, Buckwalter, Marion S, Rowitch, David H, Dawson, Valina L, Dawson, Ted M, Stevens, Beth, Barres, Ben A. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 2017 Jan 18;541(7638).
- [226] JE, Simpson, PG, Ince, G, Lace, G, Forster, PJ, Shaw, F, Matthews, G, Savva, C, Brayne, SB, Wharton. Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain - PubMed. *Neurobiology of aging* 2010 Apr;31(4).
- [227] Li, Ting, Liu, Tongtong, Chen, Xuhui, Li, Li, Feng, Miaomiao, Zhang, Yue, Wan, Li, Zhang, Chuanhan, Yao, Wenlong, Li, Ting, Liu, Tongtong, Chen, Xuhui, Li, Li, Feng, Miaomiao, Zhang, Yue, Wan, Li, Zhang, Chuanhan, Yao, Wenlong. Microglia induce the transformation of A1/A2 reactive astrocytes via the CXCR7/PI3K/Akt pathway in chronic post-surgical pain. *Journal of Neuroinflammation* 2020 17:1 2020-07-14;17(1).
- [228] Tanga, F.Y, Raghavendra, V, DeLeo, J.A. Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochemistry International* 2004/07/01;45(2-3).
- [229] Takano, Takahiro, Kang, Jian, Jaiswal, Jyoti K, Simon, Sanford M, Lin, Jane H-C, Yu, Yufei, Li, Yuxing, Yang, Jay, Dienel, Gerald, Zielke, H Ronald, Nedergaard, Maiken. Receptor-mediated glutamate release from volume sensitive channels in astrocytes. *P Natl Acad Sci USA* 2005 Oct 27;102(45).
- [230] H, Luo, HZ, Liu, WW, Zhang, M, Matsuda, N, Lv, G, Chen, ZZ, Xu, YQ, Zhang. Interleukin-17 Regulates Neuron-Glial Communications, Synaptic Transmission, and Neuropathic Pain after Chemotherapy - PubMed. *Cell reports* 11/19/2019;29(8).

- [231] Jiang, Xiaojuan, Zhou, Ruihao, Zhang, Yujun, Zhu, Tao, Li, Qian, Zhang, Weiyi. Interleukin-17 as a potential therapeutic target for chronic pain. *Frontiers in Immunology* 2022 Sep 29;13.
- [232] Yi, Min-Hee, Liu, Yong U, Liu, Kevin, Chen, Tingjun, Bosco, Dale B, Zheng, Jiaying, Xie, Manling, Zhou, Lijun, Qu, Wenchun, Wu, Long-Jun. Chemogenetic manipulation of microglia inhibits neuroinflammation and neuropathic pain in mice. *Brain, behavior, and immunity* 2020 Nov 20;92.
- [233] Fumagalli, Giulia, Monza, Laura, Cavaletti, Guido, Rigolio, Roberta, Merregalli, Cristina. Neuroinflammatory Process Involved in Different Preclinical Models of Chemotherapy-Induced Peripheral Neuropathy. *Frontiers in Immunology* 2021 Feb 4;11.
- [234] Chen, Xue-Tai, Chen, Li-Ping, Fan, Li-Jun, Kan, Hou-Ming, Wang, Zi-Zhu, Qian, Bin, Pan, Zhi-Qiang, Shen, Wen. Microglial P2Y<sub>12</sub> Signaling Contributes to Cisplatin-induced Pain Hypersensitivity via IL-18-mediated Central Sensitization in the Spinal Cord. *The Journal of Pain* 2023/05/01;24(5).
- [235] Robinson, Caleb R, Zhang, Hongmei, Dougherty, Patrick M. Astrocytes, but not microglia, are activated in oxaliplatin and bortezomib-induced peripheral neuropathy in the rat. *Neuroscience* 2014 Jun 4;274.
- [236] YR, Wen, MR, Suter, RR, Ji, GC, Yeh, YS, Wu, KC, Wang, T, Kohno, WZ, Sun, CC, Wang. Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia - PubMed. *Anesthesiology* 2009 Jan;110(1).
- [237] Kao, Sheng-Chin, Zhao, Xiuli, Lee, Chun-Yi, Atianjoh, Fidelis E, Gauda, Estelle B, Yaster, Myron, Tao, Yuan-Xiang. Absence of mu opioid receptor mRNA expression in astrocytes and microglia of rat spinal cord. *Neuroreport* 2012 Apr 18;23(6).
- [238] Lewis, Susannah S, Hutchinson, Mark R, Rezvani, Niloofer, Loram, Lisa C, Zhang, Yingning, Maier, Steven F, Rice, Kenner C, Watkins, Linda R. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1 $\beta$ . *Neuroscience* 2010 Jan 20;165(2).
- [239] Watkins, Linda R, Hutchinson, Mark R, Rice, Kenner C, Maier, Steven F. The "Toll" of Opioid-Induced Glial Activation: Improving the Clinical Efficacy of Opioids by Targeting Glia. *Trends in pharmacological sciences* 2009 Sep 15;30(11).
- [240] Green, Jacob M, Sundman, Mark H, Chou, Ying-hui. Opioid-Induced Microglia Reactivity Modulates Opioid Reward, Analgesia, and Behavior. *Neuroscience and biobehavioral reviews* 2022 Jan 25;135.
- [241] Boyette-Davis, Jessica A, Walters, Edgar T, Dougherty, Patrick M. Mechanisms involved in the development of chemotherapy-induced neuropathy. *Pain management* 2015 Jun 19;5(4).
- [242] Santin, Giada, Scietti, Luigi, Veneroni, Paola, Barni, Sergio, Bernocchi, Graziella, Bottone, Maria Grazia. Effects of Cisplatin in Neuroblastoma Rat Cells: Damage to Cellular Organelles. *International Journal of Cell Biology* 2012 Feb 28;2012.
- [243] M, Takeda, T, Tanimoto, J, Kadoi, M, Nasu, M, Takahashi, J, Kitagawa, S, Matsumoto. Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation - PubMed. *Pain* 2007 May;129(1-2).
- [244] EK, Joseph, JD, Levine. Caspase signalling in neuropathic and inflammatory pain in the rat - PubMed. *The European journal of neuroscience* 2004 Dec;20(11).

- [245] JJ, Lee, SM, Swain. Peripheral neuropathy induced by microtubule-stabilizing agents - PubMed. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 04/01/2006;24(10).
- [246] Kim, Leejeong, Nan, Guanghai, Kim, Hee Young, Cha, Myeounghoon, Lee, Bae Hwan. Modulation of chemotherapy-induced peripheral neuropathy by JZL195 through glia and the endocannabinoid system. *Biomedicine & Pharmacotherapy* 2024/11/01;180.
- [247] Zhang, Haijun, Boyette-Davis, Jessica A, Kosturakis, Alyssa K, Li, Yan, Yoon, Seo-Yeon, Walters, Edgar T, Dougherty, Patrick M. Induction of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy. *The journal of pain : official journal of the American Pain Society* 2013 May 31;14(10).
- [248] Kierdorf, Katrin, Prinz, Marco. Factors regulating microglia activation. *Frontiers in Cellular Neuroscience* 2013 Apr 23;7.
- [249] K, Biber, H, Neumann, K, Inoue, HW, Boddeke. Neuronal 'On' and 'Off' signals control microglia - PubMed. *Trends in neurosciences* 2007 Nov;30(11).
- [250] AE, Cardona, EP, Pioro, ME, Sasse, V, Kostenko, SM, Cardona, IM, Dijkstra, D, Huang, G, Kidd, S, Dombrowski, R, Dutta, JC, Lee, DN, Cook, S, Jung, SA, Lira, DR, Littman, RM, Ransohoff. Control of microglial neurotoxicity by the fractalkine receptor - PubMed. *Nature neuroscience* 2006 Jul;9(7).
- [251] D, Davalos, J, Grutzendler, G, Yang, JV, Kim, Y, Zuo, S, Jung, DR, Littman, ML, Dustin, WB, Gan. ATP mediates rapid microglial response to local brain injury in vivo - PubMed. *Nature neuroscience* 2005 Jun;8(6).
- [252] Garrido-Mesa, N, Zarzuelo, A, Gálvez, J. Minocycline: far beyond an antibiotic. *British Journal of Pharmacology* 2013 Apr 25;169(2).
- [253] Kielian, Tammy, Esen, Nilufer, Liu, Shuliang, Phulwani, Nirmal K, Syed, Mohsin M, Phillips, Napoleon, Nishina, Koren, Cheung, Ambrose L, Schwartzman, Joseph D, Ruhe, Jorg J. Minocycline Modulates Neuroinflammation Independently of Its Antimicrobial Activity in Staphylococcus aureus-Induced Brain Abscess. *The American Journal of Pathology* 2007 Oct;171(4).
- [254] D, Zemke, A, Majid. The potential of minocycline for neuroprotection in human neurologic disease - PubMed. *Clinical neuropharmacology* 2004 Nov-Dec;27(6).
- [255] Blecharz-Lang, Kinga G, Patsouris, Victor, Nieminen-Kelhä, Melina, Seiffert, Stefanie, Schneider, Ulf C, Vajkoczy, Peter. Minocycline Attenuates Microglia/Macrophage Phagocytic Activity and Inhibits SAH-Induced Neuronal Cell Death and Inflammation. *Neurocritical Care* 2022 May 18;37(2).
- [256] Kobayashi, K, Imagama, S, Ohgomori, T, Hirano, K, Uchimura, K, Sakamoto, K, Hirakawa, A, Takeuchi, H, Suzumura, A, Ishiguro, N, Kadomatsu, K, Kobayashi, K, Imagama, S, Ohgomori, T, Hirano, K, Uchimura, K, Sakamoto, K, Hirakawa, A, Takeuchi, H, Suzumura, A, Ishiguro, N, Kadomatsu, K. Minocycline selectively inhibits M1 polarization of microglia. *Cell Death & Disease* 2013 4:3 2013-03-07;4(3).
- [257] Nikodemova, Maria, Watters, Jyoti J., Jackson, Samuel J., Yang, Shaun K., Duncan, Ian D. Minocycline Down-regulates MHC II Expression in Microglia and Macrophages through Inhibition of IRF-1 and Protein Kinase C (PKC) $\alpha/\beta$ II. *Journal of Biological Chemistry* 2007/05/18;282(20).

- [258] Shin, Dong Ah, Kim, Tae Uk, Chang, Min Cheol. Minocycline for Controlling Neuropathic Pain: A Systematic Narrative Review of Studies in Humans. *Journal of Pain Research* 2021 Jan 26;14.
- [259] YQ, Zhou, DQ, Liu, SP, Chen, J, Sun, XM, Wang, YK, Tian, W, Wu, DW, Ye. Minocycline as a promising therapeutic strategy for chronic pain - PubMed. *Pharmacological research* 2018 Aug;134.
- [260] Chen, Gang, Zhang, Yu-Qiu, Qadri, Yawar J, Serhan, Charles N, Ji, Ru-Rong. Microglia in Pain: Detrimental and protective roles in pathogenesis and resolution of pain. *Neuron* 2018 Dec 19;100(6).
- [261] Martin, Laurent F, Cheng, Kevin, Washington, Stephanie M, Denton, Millie, Goel, Vasudha, Khandekar, Maithili, Largent-Milnes, Tally M, Patwardhan, Amol, Ibrahim, Mohab M. Green light exposure elicits anti-inflammation, endogenous opioid release and dampens synaptic potentiation to relieve post-surgical pain. *The journal of pain* 2022 Oct 23;24(3).
- [262] Yuan, Tian, Manohar, Krishna, Latorre, Rocco, Orock, Albert, Meerveld, Beverley Greenwood-Van. Inhibition of Microglial Activation in the Amygdala Reverses Stress-Induced Abdominal Pain in the Male Rat. *Cellular and Molecular Gastroenterology and Hepatology* 2020/01/01;10(3).
- [263] Starobova, H., Mueller, A., Allavena, R., Lohman, R. J., Sweet, M. J., Vetter, I. *Frontiers | Minocycline Prevents the Development of Mechanical Allodynia in Mouse Models of Vincristine-Induced Peripheral Neuropathy. Frontiers in Neuroscience* 2019/06/27;13.
- [264] Nazemi, Samad, Manaheji, Homa, Noorbakhsh, Syed Mohammad, Zaringhalam, Jalal, Sadeghi, Mehdi, Mohammad-Zadeh, Mohammad, Haghparast, Abbas. Inhibition of microglial activity alters spinal wide dynamic range neuron discharge and reduces microglial Toll-like receptor 4 expression in neuropathic rats. *Clinical and Experimental Pharmacology and Physiology* 2015/07/01;42(7).
- [265] Loprinzi, Charles L., Lacchetti, Christina, Bleeker, Jonathan, Cavaletti, Guido, Chauhan, Cynthia, Hertz, Daniel L., Kelley, Mark R., Lavino, Antoinette, Lustberg, Maryam B., Paice, Judith A., Schneider, Bryan P., Smith, Ellen M. Lavoie, Smith, Mary Lou, Smith, Thomas J., Wagner-Johnston, Nina, Hershman, Dawn L. Prevention and Management of Chemotherapy-Induced Peripheral Neuropathy in Survivors of Adult Cancers: ASCO Guideline Update. *Journal of Clinical Oncology* 2020-10-1;38(28).
- [266] XS, Wang, Q, Shi, NA, Bhadkamkar, CS, Cleeland, A, Garcia-Gonzalez, JR, Aguilar, C, Heijnen, C, Eng. Minocycline for Symptom Reduction During Oxaliplatin-Based Chemotherapy for Colorectal Cancer: A Phase II Randomized Clinical Trial - PubMed. *Journal of pain and symptom management* 2019 Oct;58(4).
- [267] DR, Pachman, T, Dockter, PJ, Zekan, B, Fruth, KJ, Ruddy, LE, Ta, JM, Lafky, T, Dentchev, NA, Le-Lindqwister, WM, Sikov, N, Staff, AS, Beutler, CL, Loprinzi. A pilot study of minocycline for the prevention of paclitaxel-associated neuropathy: ACCRU study RU221408I - PubMed. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer* 2017 Nov;25(11).
- [268] Schlesinger, Naomi, Meulemeester, Marc De, Pikhlak, Andrey, Yücel, A Eftal, Richard, Dominik, Murphy, Valda, Arulmani, Udayasankar, Sallstig, Peter, So, Alexander. Canakinumab relieves symptoms of acute flares and improves health-related quality of life

- in patients with difficult-to-treat Gouty Arthritis by suppressing inflammation: results of a randomized, dose-ranging study. *Arthritis Research & Therapy* 2011 Mar 25;13(2).
- [269] Betrains, A, Staels, F, Vanderschueren, S. Efficacy and safety of canakinumab treatment in schnitzler syndrome: A systematic literature review. *Seminars in Arthritis and Rheumatism* 2020/08/01;50(4).
- [270] Kobayashi, Masaaki, Konishi, Hiroyuki, Sayo, Akira, Takai, Toshiyuki, Kiyama, Hiroshi. TREM2/DAP12 Signal Elicits Proinflammatory Response in Microglia and Exacerbates Neuropathic Pain. *The Journal of Neuroscience* 2016 Oct 26;36(43).
- [271] Khomeijani-Farahani, Mohammadreza, karami, Jafar, Farhadi, Elham, Soltani, Samaneh, Delbandi, Ali-Akbar, Shekarabi, Mehdi, Tahmasebi, Mohammad Naghi, Vaziri, Arash Sharafat, Jamshidi, Ahmadreza, Mahmoudi, Mahdi, Akhlaghi, Masoomeh, Khomeijani-Farahani, Mohammadreza, karami, Jafar, Farhadi, Elham, Soltani, Samaneh, Delbandi, Ali-Akbar, Shekarabi, Mehdi, Tahmasebi, Mohammad Naghi, Vaziri, Arash Sharafat, Jamshidi, Ahmadreza, Mahmoudi, Mahdi, Akhlaghi, Masoomeh. TAK-242 (Resatorvid) inhibits proinflammatory cytokine production through the inhibition of NF- $\kappa$ B signaling pathway in fibroblast-like synoviocytes in osteoarthritis patients. *Advances in Rheumatology* 2024 64:1 2024-06-07;64(1).
- [272] Henry, Rebecca J, Ritzel, Rodney M, Barrett, James P, Doran, Sarah J, Jiao, Yun, Leach, Jennie B, Szeto, Gregory L, Wu, Junfang, Stoica, Bogdan A, Faden, Alan I, Loane, David J. Microglial Depletion with CSF1R Inhibitor During Chronic Phase of Experimental Traumatic Brain Injury Reduces Neurodegeneration and Neurological Deficits. *The Journal of Neuroscience* 2020 Apr 1;40(14).
- [273] Claeyss, Wouter, Verhaege, Daan, Van Imschoot, Griet, Van Wonterghem, Elien, Van Acker, Lore, Amelinck, Laura, De Ponti, Federico F., Scott, Charlotte, Geerts, Anja, Van Steenkiste, Christophe, Van Hoecke, Lien, Vandenbroucke, Roosmarijn E. *Frontiers | Limitations of PLX3397 as a microglial investigational tool: peripheral and off-target effects dictate the response to inflammation. Frontiers in Immunology* 2023/11/22;14.
- [274] Sawicki, Caroline M., Kim, January K., Weber, Michael D., Faw, Timothy D., McKim, Daniel B., Madalena, Kathryn M., Lerch, Jessica K., Basso, D. Michele, Humeidan, Michelle L., Godbout, Jonathan P., Sheridan, John F. Microglia Promote Increased Pain Behavior through Enhanced Inflammation in the Spinal Cord during Repeated Social Defeat Stress. *Journal of Neuroscience* 2019-02-13;39(7).
- [275] Juni, A., Klein, G., Pintar, J.E., Kest, B. Nociception increases during opioid infusion in opioid receptor triple knock-out mice. *Neuroscience* 2007/06/29;147(2).
- [276] Watkins, Linda R., Hutchinson, Mark R., Johnston, Ian N., Maier, Steven F. Glia: novel counter-regulators of opioid analgesia. *Trends in Neurosciences* 2005/12/01;28(12).
- [277] Eidson, Lori N., Murphy, Anne Z. Inflammatory mediators of opioid tolerance: Implications for dependency and addiction. *Peptides* 2019/05/01;115.
- [278] Araldi, Dioneia, Bogen, Oliver, Green, Paul G, Levine, Jon D. Role of Nociceptor Toll-like Receptor 4 (TLR4) in Opioid-Induced Hyperalgesia and Hyperalgesic Priming. *The Journal of Neuroscience* 2019 Aug 14;39(33).
- [279] Shah, Masaud, Choi, Sangdun. Toll-like Receptor-Dependent Negative Effects of Opioids: A Battle between Analgesia and Hyperalgesia. *Frontiers in Immunology* 2017 May 31;8.

- [280] Benjamini, Dan, Priemer, David S, Perl, Daniel P, Brody, David L, Basser, Peter J. Mapping astrogliosis in the individual human brain using multidimensional MRI. *Brain* 2022 Aug 12;146(3).
- [281] Escartin, Carole, Galea, Elena, Lakatos, András, O’Callaghan, James P, Petzold, Gabor C, Serrano-Pozo, Alberto, Steinhäuser, Christian, Volterra, Andrea, Carmignoto, Giorgio, Agarwal, Amit, Allen, Nicola J, Araque, Alfonso, Barbeito, Luis, Barzilai, Ari, Bergles, Dwight E, Bonvento, Gilles, Butt, Arthur M, Chen, Wei-Ting, Cohen-Salmon, Martine, Cunningham, Colm, Deneen, Benjamin, Strooper, Bart De, Díaz-Castro, Blanca, Farina, Cinthia, Freeman, Marc, Gallo, Vittorio, Goldman, James E, Goldman, Steven A, Götz, Magdalena, Gutiérrez, Antonia, Haydon, Philip G, Heiland, Dieter H, Hol, Elly M, Holt, Matthew G, Iino, Masamitsu, Kastanenka, Ksenia V, Kettenmann, Helmut, Khakh, Baljit S, Koizumi, Schuichi, Lee, C Justin, Liddelow, Shane A, MacVicar, Brian A, Magistretti, Pierre, Messing, Albee, Mishra, Anusha, Molofsky, Anna V, Murai, Keith K, Norris, Christopher M, Okada, Seiji, Oliet, Stéphane H R, Oliveira, João F, Panatier, Aude, Parpura, Vladimir, Pekna, Marcela, Pekny, Milos, Pellerin, Luc, Perea, Gertrudis, Pérez-Nievas, Beatriz G, Pfrieger, Frank W, Poskanzer, Kira E, Quintana, Francisco J, Ransohoff, Richard M, Riquelme-Perez, Miriam, Robel, Stefanie, Rose, Christine R, Rothstein, Jeffrey D, Rouach, Nathalie, Rowitch, David H, Semyanov, Alexey, Sirko, Svetlana, Sontheimer, Harald, Swanson, Raymond A, Vitorica, Javier, Wanner, Ina-Beate, Wood, Levi B, Wu, Jiaqian, Zheng, Binhai, Zimmer, Eduardo R, Zorec, Robert, Sofroniew, Michael V, Verkhratsky, Alexei. Reactive astrocyte nomenclature, definitions, and future directions. *Nature neuroscience* 2021 Feb 15;24(3).
- [282] L, Facci, M, Barbierato, SD, Skaper. Astrocyte/Microglia Cocultures as a Model to Study Neuroinflammation - PubMed. *Methods in molecular biology (Clifton, NJ)* 2018;1727.
- [283] Prato, Vincenzo, Taberner, Francisco J, Hockley, James RF, Callejo, Gerard, Arcourt, Alice, Tazir, Bassim, Hammer, Leoni, Schad, Paulina, Heppenstall, Paul A, Smith, Ewan St John, Lechner, Stefan G. “Genetic identification of mechanoinensitive ‘silent’ nociceptors”. *Cell reports* 2017 Dec 12;21(11).
- [284] M, Michaelis, HJ, Häbler, W, Jäenig. Silent afferents: a separate class of primary afferents? - PubMed. *Clinical and experimental pharmacology & physiology* 1996 Feb;23(2).
- [285] Das Gupta, Rebecca Rani, Scheurer, Louis, Pelczar, Pawel, Wildner, Hendrik, Zeilhofer, Hanns Ulrich, Das Gupta, Rebecca Rani, Scheurer, Louis, Pelczar, Pawel, Wildner, Hendrik, Zeilhofer, Hanns Ulrich. Neuron-specific spinal cord translatomes reveal a neuropeptide code for mouse dorsal horn excitatory neurons. *Scientific Reports* 2021 11:1 2021-03-04;11(1).
- [286] S, Zeng, Y, Liang, H, Hu, F, Wang, L, Liang. Endothelial cell-derived S1P promotes migration and stemness by binding with GPR63 in colorectal cancer - PubMed. *Pathology, research and practice* 2022 Dec;240.
- [287] E, Kostenis. Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine, and (lyso)-phosphatidic acid: new receptors for "old" ligands - PubMed. *Journal of cellular biochemistry* 08/01/2004;92(5).
- [288] H, Yin, A, Chu, W, Li, B, Wang, F, Shelton, F, Otero, DG, Nguyen, JS, Caldwell, YA, Chen. Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay - PubMed. *The Journal of biological chemistry* 05/01/2009;284(18).

- [289] Gloriam, David E.I., Schiöth, Helgi B., Fredriksson, Robert. Nine new human Rhodopsin family G-protein coupled receptors: identification, sequence characterisation and evolutionary relationship. *Biochimica et Biophysica Acta (BBA) - General Subjects* 2005/04/15;1722(3).
- [290] Barnea, Gilad, Strapps, Walter, Herrada, Gilles, Berman, Yemiliya, Ong, Jane, Kloss, Brian, Axel, Richard, Lee, Kevin J. The genetic design of signaling cascades to record receptor activation. *P Natl Acad Sci USA* 2007 Dec 28;105(1).
- [291] Kroeze, WK, Sassano, MF, Huang, X-P, Lansu, K, McCorvy, JD, Giguere, PM, Sciaky, N, Roth, BL. PRESTO-TANGO: an open-source resource for interrogation of the druggable human GPCR-ome. *Nature structural & molecular biology* 2015 Apr 20;22(5).
- [292] Zanden, Sabina Y. van der, Qiao, Xiaohang, Neefjes, Jacques. *FEBS Press. The FEBS Journal* 2021/11/01;288(21).
- [293] Demain, Arnold L, Sanchez, Sergio. Microbial drug discovery: 80 years of progress. *The Journal of Antibiotics* 2009 Jan 9;62(1).
- [294] BL, Ligon. Penicillin: its discovery and early development - PubMed. *Seminars in pediatric infectious diseases* 2004 Jan;15(1).
- [295] Caen, Ouriel, Schütz, Simon, Jammalamadaka, M. S. Suryateja, Vrignon, Jérémy, Nizard, Philippe, Schneider, Tobias M., Baret, Jean-Christophe, Taly, Valérie, Caen, Ouriel, Schütz, Simon, Jammalamadaka, M. S. Suryateja, Vrignon, Jérémy, Nizard, Philippe, Schneider, Tobias M., Baret, Jean-Christophe, Taly, Valérie. High-throughput multiplexed fluorescence-activated droplet sorting. *Microsystems & Nanoengineering* 2018 4:1 2018-10-22;4(1).