CENTRAL SENSORIMOTOR CHANGES DUE TO PERIPHERAL NERVE INJURY: ROLE OF DOPAMINERGICS AND OPIOIDS

By

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ABSTRACT

Chronic neuropathic pain (CNP) is a disease typically resulting from injury to the central or peripheral nervous system. CNP has a prevalence of 12-15% in the population, but current treatments are not highly efficacious in achieving long term analgesia. Chronic use of opioids is a typical but dangerous regimen that usually requires high doses to achieve analgesia, putting patients at elevated risk for opioid overdose and negative side effects.

We have previously shown that adjuvant administration of a dopamine (DA) D3 receptor agonist, pramipexole (PPX), with morphine achieved analgesia in in a centrally induced model of CNP. Here we used a more clinically relevant disease of peripherally induced CNP (sciatic nerve ligation, SNL) to test if this treatment would be more effective than morphine. To test these hypotheses, male mice (C57BL/6) underwent a unilateral SNL, and thermal pain withdrawal reflex latencies (TPWRLs) were measured on injured and uninjured sides under control and drug treatment conditions. We found that neither morphine nor PPX alone restored TPWRLs, which are decreased after SNL, but that administering both drugs as an adjuvant therapy (morphine + PPX) under acute conditions led to a synergistic effect that fully restored

TPWRLs to pre-injury baseline levels. With this approach, a full analgesic effect was observed even after reducing the morphine dose by 50%.

Next, we tested if continuous daily treatment with the adjuvant (morphine + PPX) at its lower dose could maintain analgesia over time. We found that chronic treatment with the combination mimicked the acute effects, in that it restored and maintained TPWRLs on the SNL side at levels similar to those of the contralateral un-injured sides, and effect was maintained over time. Animals showed no sign of recovery from injury, or tolerance to treatment.

In addition, we tested with extracellular electrophysiology for the effects of SNL and subsequent drug treatments on compound action potentials (CAPs) in injured and un-injured sciatic nerves. To test this, we conducted a series of analyses and found that CAPs of injured nerves were consistently lower than those of their contralateral controls. We found that CAPs were lost after SNL and that they did not recover after prolonged treatment with either morphine or PPX, but that they showed a trend towards a recovery after long-term treatment with the drug combination.

In conclusion, our data indicate adjuvant treatment of a D3 agonist with morphine can achieve and maintain analgesia over time, and this effect may be mediated in part by changes in the injured sciatic nerves themselves. Combining these data with our previous studies suggests that this adjuvant may serve as a new pharmacological treatment for CNP regardless of its origin.

Ultimately, these findings may lead to the development of a novel effective method of pain relief that reduces negative side effects of using high dose opioids, and they may contribute to a potential reduction of the opioid epidemic.

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Mandee Kate Schaub

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I dedicate this work to Mason Michael Futrell, and Tyler Joy Strong. May there be a safe and efficacious treatment for chronic neuropathic pain well before you experience any.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CHAPTER 1: Introduction

1.1 Chronic Pain Overview

Chronic pain is a primary reason patients seek medical care and can present across a wide range of disease states including migraines, cancer, and arthritis. Chronic pain affects more than 30% of people worldwide and is defined as pain that lasts more than 3 months, regardless of origin and location (Schappert and Burt 2006, Cohen, Vase and Hooten 2021). Chronic pain can be further categorized as either nociceptive, or neuropathic (Scholz 2014). Nociceptive chronic pain is identified as a response of nociceptors to a noxious stimulus that may result from an injury to non-neural body tissues, either superficial like skin, or deep body tissues, such as muscles or joints (Smart et al. 2010). In contrast, an injury to the nervous system, either the central nervous system (**CNS**) or the peripheral nervous system (**PNS**) can result in neuropathic pain. Neuropathic pain affects 30-40% of all chronic pain patients and may present symptoms such as shooting pains, tingling, numbness, or a feeling of pins and needles (van Velzen, Dahan and Niesters 2020).

1.2 Mechanisms Underlying Chronic Pain

A major hallmark of pain is the response from different receptors to a noxious stimulus. Pain stimuli can be classified by their origin as mechanical (pressure or pinch), thermal (hot or cold sensing) and chemical pain (inflammation, ischemia, or infection) (Campbell and Meyer 2006, Cohen and Raja 2012). These pain stimuli are converted to electrical signals that are transmitted by specific afferent nerve fibers (Yam et al. 2018). These sensory nerve fibers are either

unmyelinated C-fibers (conduction velocity ~ 0.05-2 m/s), or thinly myelinated Aδ-fibers (conduction velocities ~ 3-30 m/s) (Cohen and Raja 2012). Functionally, C-fibers transmit thermal signals, while Ad-fibers transmit mechanical and chemical signals (Millan 1999). Both nociceptors are pseudo-unipolar neurons whose cell bodies are located in the dorsal root ganglia (**DRG**) adjacent to the spinal cord (Meltzer et al. 2021). Their afferent projections enter the spinal cord at the dorsal horn where they synapse onto pain-processing sensory neurons, and collateral projections carry the signals to the thalamus, which processes the signals and sends them to the somatosensory cortex (Baron, Binder and Wasner 2010, Cohen and Raja 2012). The brain then interprets the pain signal and adds a affective component of pain (Meltzer et al. 2021). A simplified model of pain circuitry is shown in Figure 1. It highlights both peripheral (spinal cord) and central (brain) CNS regions that are involved in pain processing.

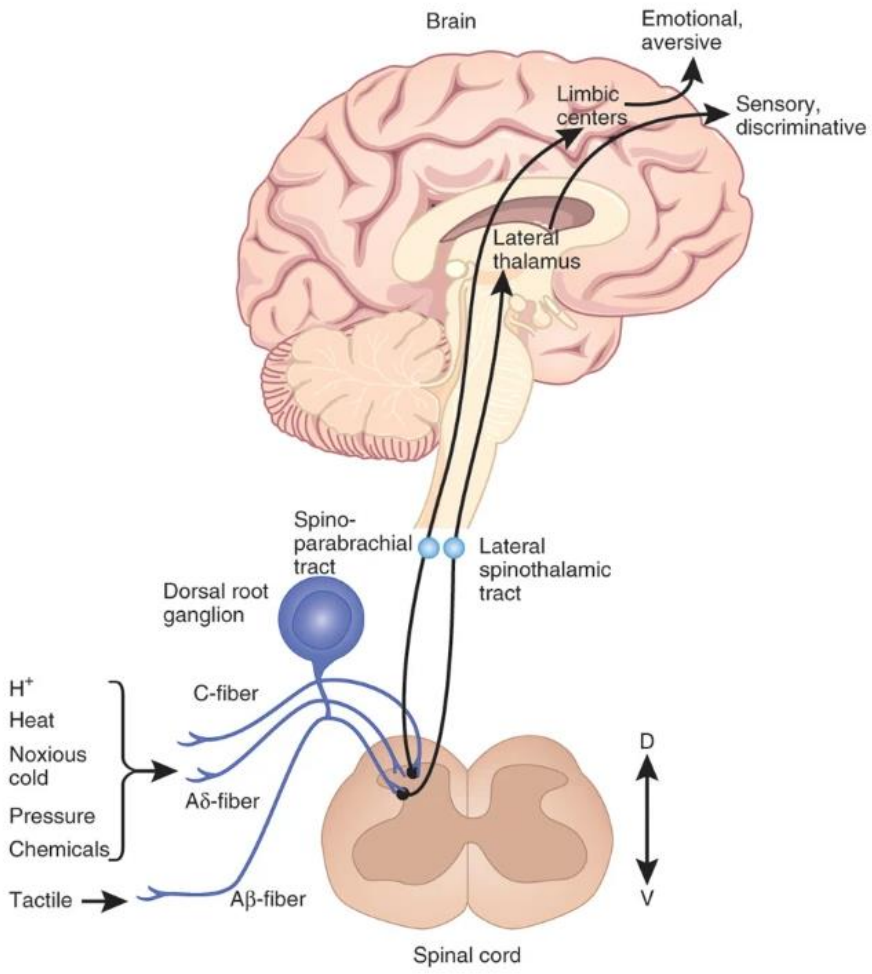

Figure 1: A schematic overview of the main circuits mediating physiological pain. Noxious stimuli of various modalities are sensed by a specialized set of nerve fibers: unmyelinated C fibers and thinly myelinated Aδ fibers, which are distinct from myelinated tactile sensors (Aβ fibers) and proprioceptors. The physicochemical properties of noxious stimuli, such as heat, extreme cold, pressure and chemicals, are converted to electrical activity, and this electrical activity is amplified by sodium channels to elicit action potentials. Nociceptive afferents carrying these peripheral inputs form glutamatergic synapses onto second-order neurons mostly in the superficial laminae (I and II) in the spinal dorsal horn, whereas inputs from non-nociceptive fibers form synapses in deeper laminae. Some integration and processing of sensory inputs occurs in the spinal dorsal horn, and the net output from spinal networks is carried by several pathways to distinct projection sites in the brain. For example, the lateral spinothalamic tract projects multimodal sensory inputs from spinal wide-dynamic range neurons to the lateral thalamus and has been implicated in processing sensory and discriminative aspects of pain. By contrast, the medial aspect of the spinothalamic tract and the spinoparabrachial tract project to the medial thalamus and limbic structures and are believed to mediate the emotional and aversive components of pain. The experience of pain is perceived in the cortex, and information is accordingly sent to the spinal cord to enable withdrawal from the noxious stimulus. From: (Kuner 2010)

Known peripheral mechanisms of neuropathic pain include abnormal spontaneous activity categorized as ectopic discharges in nociceptive fibers (Attal 2000). These ectopic discharges involve dysregulation of the synthesis and spatial redistribution of the sodium channels that control membrane excitability. Tetrodotoxin (**TTX**)-resistant sodium channels, which are only found on nociceptor sensory neurons (Tan et al. 2014), have been found to accumulate at the site of neuropathic injury (Novakovic et al. 1998). Following peripheral nerve injury, decreased levels of GABA and glycine, and downregulation of GABA receptors have been reported at the spinal dorsal horn (Attal 2000). Modulation of GABA mediated pathways have been shown to influence pain responses in patients. Peripheral nerve damage can also lead to a reorganization of primary afferents in the spinal cord (Woolf 1997). Large diameter primary afferents that transfer nociceptive tactile messages and normally terminate in Laminae III and IV can in chronic pain conditions extend into Lamina II, where input is usually received from small diameter fibers responding to noxious stimuli (Woolf 1997). Thus, these nociceptive neurons in Lamina II are then activated by non-noxious stimulation, leading to pain sensation. Abnormal hyperexcitability of central nociceptive neurons, known as central sensitization, is an example of one mechanism of central neuropathic pain (Attal 2000). This pathway is dependent on N-methyl-D aspartate (**NMDA**) glutaminergic receptors activated by an abundant release of excitatory amino acids, glutamate, and aspartate. Activation of NMDA receptors have been shown to produce a local CNS analgesic effect. These receptors are located on the membrane of spinal dorsal horn neurons (Yang et al. 2015). Central disinhibition is the abnormal excitability of central neurons, resulting from losing modulatory control mechanisms (Attal 2000). In the

case of a central disinhibition, lateral and medial pain pathways become dysbalanced and neural firing thresholds are lowered (Forstenpointner et al. 2020).

1.3 Chronic Neuropathic Pain

Chronic Neuropathic Pain (**CNP**) can derive from lesions or diseases of the central or peripheral nervous system (CNS or PNS, respectively) (Attal 2000) and effects 12-15% of the US population (Colloca et al. 2017). CNP is typically a consequence of spinal cord injury, stroke, multiple sclerosis, but can arise from many conditions affecting the CNS (Widerström-Noga et al. 2017). Peripheral nerve injuries are classified as polyneuropathies, mononeuropathies, and multiple mononeuropathies. Polyneuropathies are the most common type of nervous system disorder in adults, and affect multiple nerves of the PNS (Sommer et al. 2018). While the underlying mechanisms are often unclear, polyneuropathies are most commonly associated with diseases such as diabetes, alcohol use disorder, and chemotherapy treatments (Sommer et al. 2018). Mononeuropathies are associated with a single nerve in the PNS and are most commonly associated with local injury (Dewey and Talarico 2008). With an incidence of 10% in adults over 30, CNP is one of the most difficult pain diseases to treat, with most patients rating their pain management as unsatisfactory (van Hecke et al. 2014). Moreover, CNP is 50% more likely than nociceptive pain to worsen over time (Watson and Sandroni 2016).

1.4 Defining the Subtypes of Chronic Neuropathic Pain

Centrally induced neuropathic pain (**CINP**), which develops as a result of injury to the CNS, can arise from multiple diseases and/or injuries. Some specific CINP syndromes include central post

stroke pain, spinal cord injury pain, multiple sclerosis related pain, and pain related to Parkinson's disease. Descriptors of neuropathic pain can include burning, uncomfortable cold, prickling, tingling, pins and needles, stabbing, shooting, tight, swollen, and squeezing sensations that are distressing (Dworkin 2002). Although these descriptors suggest an origin to neuropathic pain etiology, they are not specific to CINP alone (Watson and Sandroni 2016). Because CINP symptoms are difficult to distinguish from nociceptive pain and peripheral nerve pain, diagnosis focuses on the origin of the pain (i.e. disease state), if known. There are some resources for diagnosing neuropathic pain, including, the Neuropathic Pain Questionnaire (Krause and Backonja 2003), the Leeds Assessment of Neuropathic Symptoms and Signs (Bennett 2001), the Neuropathic Pain Symptom Inventory (Bouhassira et al. 2004), and the Neuropathic Pain Scale (Galer and Jensen 1997).

1.5 Peripherally Induced Neuropathic Pain

Peripherally induced neuropathic pain (**PINP**) is more common than central neuropathic pain. It is estimated that 25-30% of chronic pain patients have peripheral neuropathic pain (Hughes 2002). As a result of injury or disease to the PNS, the outcome symptoms are very similar to those of central neuropathic pain (Rull et al. 1969). While there are numerous potential causes for PINP, some common causes are diabetic neuropathy, HIV-associated neuropathy, carpal tunnel syndrome, traumatic nerve injury, and cancer neuropathy (Sommer et al. 2018). While postherpetic neuralgia involves damage to the PNS, studies show that it can result in anatomic changes in the dorsal horn, making it a mixed neuropathic pain disease of both central and peripheral consequences (Stacey 2005). Studies show that neural reorganization occurs in PINP,

meaning there may be central complications that result from a peripherally induced injury (Baron 2000).

1.6 Interventional Therapies for Chronic Neuropathic Pain

Spinal cord stimulation is a clinical approach that uses low intensity electrical stimulation of Aβ fibers to modulate and control CNP. This intervention is based on the gate control theory, which states that spinal cord has a "gate" than can block pain signals or allow them to continue to the brain. By stimulating somatosensory and proprioceptive pathways, the pain signals are blocked and do not reach the thalamic pain relay center in the brain (Cherry 2022). The most common method of spinal cord stimulation is administering a monophasic square-wave pulse ranging in frequency from 30-100 Hz resulting in paresthesia in the painful region (Yearwood et al. 2010). A newer method stimulates with 40 Hz bursts with spikes of 500 Hz and high frequency 10 kHz with sinusoidal waveforms (Russo and Van Buyten 2015). The new method provides paresthesia-free stimulation and pain relief is equivalent or better than the monophasic square wave pulse (De Ridder et al. 2013). Based on a DRG stimulation study, a 56% pain reduction with a 60% responder rate occurs with this type stimulation (Krames 2014). Further, neurostimulation of afferent fibers outside of the spinal cord includes DRG and peripheral nerve fields. Together, these studies suggest that optimization in the treatment of peripheral nerve pain may be possible (Liem et al. 2015).

Physical therapy is recommended for treating peripheral neuropathic pain however it does not treat the underlying cause of the pain (Colloca et al. 2017). Routine exercise can prevent or delay the onset of some of the most common causes of peripheral neuropathy. Increasing mobility of

areas with peripheral neuropathy may be an effective intervention to decrease symptoms (Dobson, McMillan and Li 2014).

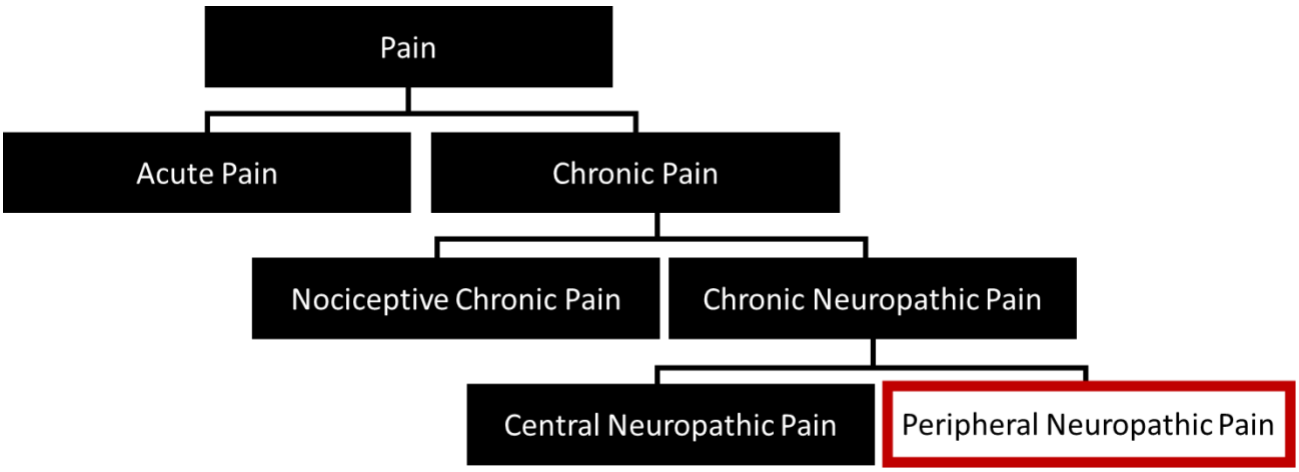

Figure 2: Overview of organization of the different aspects of pain.

1.7 Current Pharmacological Treatments for Chronic Neuropathic Pain

In neuropathic pain management, clinicians are often forced to focus on pharmacological treatment of the symptoms as the underlying etiology of the disease is not known (Colloca et al. 2017). This is further complicated, as success of pharmacological treatment for CNP varies based on the origin of the pain (Attal 2000). Pharmacological interventions to combat pain also include Tricyclic antidepressants (**TCAs**), Selective serotonin reuptake inhibitors (**SSRIs**) and others.

TCAs have shown efficacy over placebo with no significant difference between the different TCAs in comparative trials (Watson et al. 1998). TCAs work on neuropathic pain by inhibiting presynaptic reuptake of serotonin, and noradrenaline, and their NMDA and ion channel blocking action (Sindrup et al. 2005).

SSRIs have had less success than TCAs, however citalopram and paroxetine were significantly more effective than the placebo. The overall efficacy of antidepressants in CNP treatment is moderate, however due to the high side effect profile of TCAs, most patients do not reach optimal dosing for pain relief (Attal 2000).

Antiepileptic drugs have given mixed results, as they require high doses, and elicit side effects in up to 65% of patients (Eisenberg et al. 2007). Newer antiepileptics like gabapentin, lamotrigine, and topiramate show more promising results in CNP treatment than previous antiepileptics such as carbamazepine (Magnus 1999, Rowbotham et al. 1998). Gabapentin increases GABA release in the brain (Götz et al. 1993) from various brain areas (Kocsis and Honmou 1994). It works to reinforce inhibitory controls that modulate the transmission of signals of pain, and reduces production of glutamate, which is key in nociceptive signal processing (Rock, Kelly and

Macdonald 1993). Lamotrigine works by stabilizing neural membranes to reduce abnormal neuron firing, and inhibits the presynaptic release of glutamate, reducing nociceptive signaling (Eisenberg, Shifrin and Krivoy 2005). Topiramate is a third anticonvulsant that has shown efficacy in treating neuropathic pain. While the exact mechanism of action is still unknown, it is hypothesized that topiramate inhibits GABA-ergic pathways and blocks AMPA/glutamate pathways (Wiffen et al. 2013). These actions result in an increase in inhibitory neurotransmission and modulation of voltage gated ion channels to block repetitive firing of action potentials (Chong and Libretto 2003). Together, the lessons learned from the mechanism of action of antiepileptics, suggest that GABA mediated pathways may play a key role in modulating pain responses.

Local anesthetics have shown good efficacy in peripheral neuropathic pain (Attal 2000), but only a small amount of success in central neuropathic pain (Biella and Sotgiu 1993). Lidocaine blocks voltage gated sodium channels and suppresses ectopic neural discharges from injured primary afferent nerve fibers (Tanelian and MacIver 1991). These anesthetics can be applied topically (Rowbotham, Davies and Fields 1995), and have a low incidence of side effects for patients with postherpetic neuralgia and mechanical allodynia (Devers and Galer 2000).

Ketamine, an NMDA antagonist has shown short term success over placebo in many types of neuropathic pain, but that success is met with intolerable side effects preventing long-term treatment and success (Attal 2000). Ketamine blocks the calcium channel of the NMDA receptor via non-competitive antagonism, eliciting a local anesthetic action in the CNS. It also reduces presynaptic release of glutamate (Pai and Heining 2007).

1.7 Opioids as a Pharmacological Treatment for Chronic Neuropathic Pain

Opioid analgesics and opioid like analgesics are used when typical treatments are ineffective. These medications are known to have serious side effects as well as high risks for tolerance and addiction. The opioid class of drugs contains many medications, these include oxycodone, morphine, methadone, and tramadol which have shown efficacy in CNP. Of patients treated with high doses of opioids 66% reported moderate or better pain relief in the short term. However, only 7-17% were able to maintain pain relief with long term opioid therapy. 44% of patients receiving long term opioid pain management require 2 or more opioids to maintain pain relief. These mediations bind to the mu-opioid receptors and cause cellular hyperpolarization (Pathan and Williams 2012).

Despite not being highly efficacious in all types of neuropathic pain (Figure 3), opioid medications are a common neuropharmacological treatment option for neuropathic pain patients.

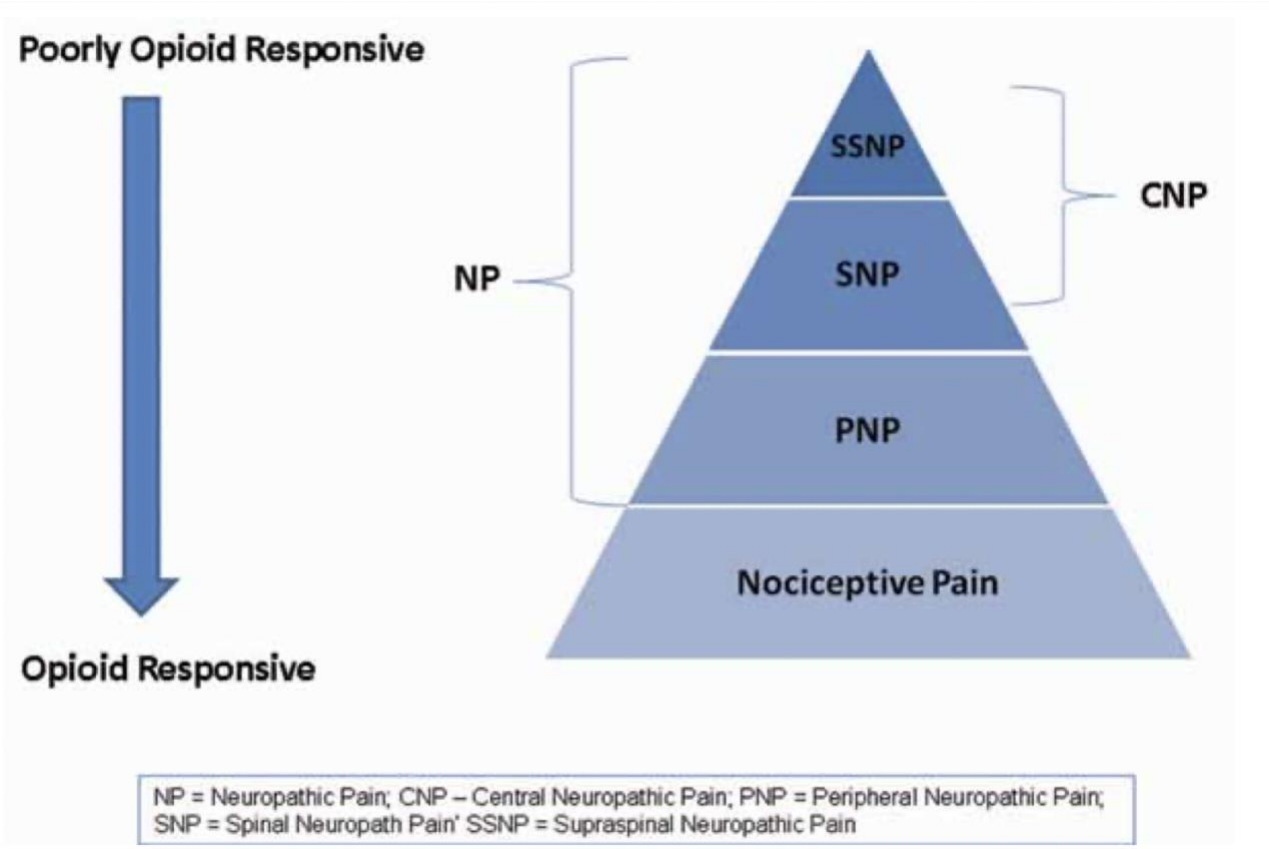

Figure 3: Different types of pain respond differently to opioid medications. This schematic shows relative opioid responsiveness of different categories of pain. The unresponsiveness of neuropathic pain to morphine highlights the need for new treatments for morphine tolerant injuries (Smith 2012).

The μ-opioid (**MOR**) receptor is one of four receptor subtypes to which opioid medications can bind. These four subtypes are the MOR, kappa-opioid receptor (**KOR**), delta-opioid receptor (**DOR**), and the nociception/orphanin FQ peptide receptor (**NOP**). There is research being done on possible analgesic effects of KOR, however side effects of hallucinations and dysphoria occur at a greater rate at doses that elicit analgesia. DORs are known to have an antidepressant and anxiolytic effect when activated, however researchers have explored using a non-selective binding agonist to activate the DOR with MORs and elicit better analgesia than only activating MORs. Experimental evidence from DOR knock out mice show that activating DORs can increase opioid tolerance nearly 5 fold, suggesting that influencing opioid receptor subtypes may influence each other (Waldhoer, Bartlett and Whistler 2004). There is no accepted role of NOR in the pain modulatory circuit, but research suggests that NOR may contribute to anti-analgesic activity (Mogil and Pasternak 2001).

The MOR is a G-protein coupled receptor (**GPCR**) located in the cell membrane. When morphine binds the MOR it leads to decreased production of cAMP and a subsequent reduction in cellular activity, which is thought to reduce pain signaling (Brewer and Clemens 2018). However, clinically-relevant side effects such as respiratory depression, euphoria, sedation, decreased gastrointestinal mobility, and physical dependance have been observed (Trescot et al. 2008). Following administration of morphine, dopamine is released in the brain from the caudate and the nucleus accumbens. The amount of dopamine released increases as the dose of morphine increases (Di Chiara and Imperato 1988). Chronic administration of morphine has shown permanent alterations in the dopamine system (Sklair-Tavron et al. 1996). The release of dopamine triggers the reward system and because morphine elicits that release, patients will continue to search for the reward produced by morphine administration (Sklair-Tavron et al.

1996). A key aspect of opioid administration is the development of tolerance. Tolerance is characterized by a reduced responsiveness to an opioid agonist. As tolerance develops, morphine doses need to be increased for the same analgesic effect to be reached (Valentino and Volkow 2018). This ultimately leads to high dose levels of opioids being prescribed to accomplish pain relief and enhances risk for dangerous side effects. These negative side effects that go along with opioid analgesia have contributed to an epidemic in the United States. This is highlighted by the Centers for Disease Control provisional prescription opioid overdose report that over 80,000 overdose deaths occurred from September 2020 - September 2021 (Ahmad, Rossen and Sutton 2021).

1.8 The Dopaminergic System and its effect on cAMP and PKA Regulation

Dopamine receptors can be grouped into several GPCR subtypes that fall into two families. The two families of dopamine receptors are D1-like, and D2-like. D1-like receptors (comprised of D1R and D5R subtypes) are thought to be activated at (relatively) higher dopamine (**DA**) concentrations, and they mediate excitatory effects via activation of adenylate cyclase (**AC**) and protein kinase A (**PKA**) pathways. They also play an important role in regulating fundamental neurophysiological processes such as mood, motivation, cognitive functions, and motor activity (Undieh 2010). In contrast, D2-like receptors (D2R, D3R, and D4R) are thought to be activated at (relatively) lower DA levels, and mediate inhibitory effects by blocking or reducing the activation of AC and PKA pathways (Fuziwara et al. 2005).

The D3R subtype has the highest affinity for DA within the D2-like family, and may be an efficient target for therapeutic agents that aim to increase inhibitory tone (Figure 4) (Clemens

and Ghorayeb 2019b). D3R agonists (pramipexole, ropinirole, rotigotine) are typical treatments for Restless Legs Syndrome (**RLS**) and Parkinson's disease (**PD**). These medications mimic the action of DA by preferably binding to D3Rs (Choi et al. 2020) thereby increasing the inhibitory effects usually mediated by low DA levels. This potential interplay between D3R and MOR may represent a novel mechanism by which pain modulation and analgesia could be influenced.

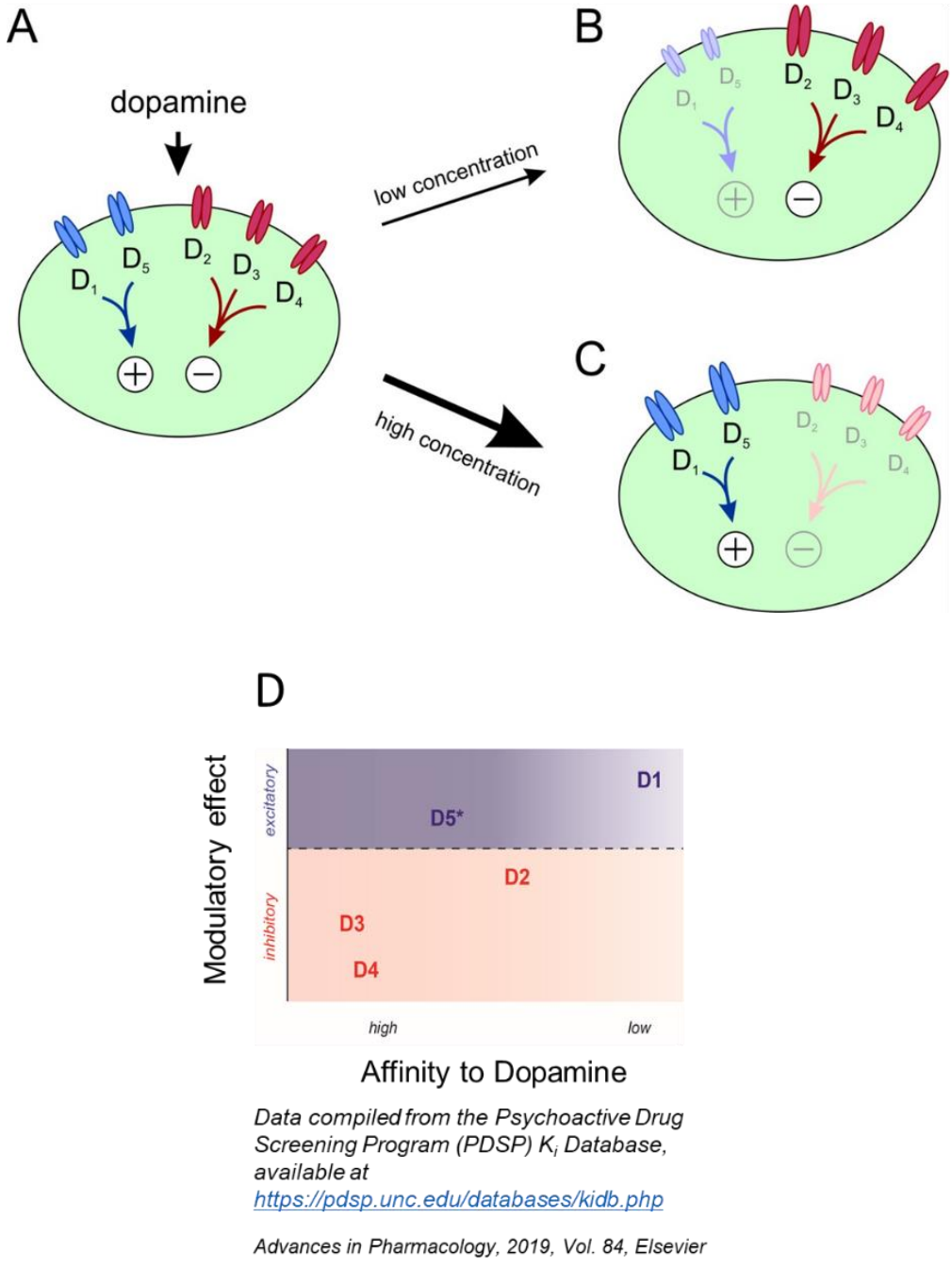

Figure 4: Dopamine receptor modulatory activity and binding affinity to substrates. **A.** Dopamine receptor subtypes and actions as inhibitory and excitatory. **B.** D2-like actions are thought to be primarily activated at low levels of DA. **C.** D2-like actions are thought to be primarily activated at low levels of DA. **D.** Receptor subtypes and their relative binding affinity to dopamine.

1.9 Main Hypothesis

The MOR is the primary opioid receptor subtype that is responsible for analgesia. A key MOR function is its ability to inhibit the production of cAMP (Figure 5, panel A). The D3 receptor has the highest affinity for DA and elicits inhibitory effects on cAMP processing Figure 5, panel B). These underlying mechanisms of action on cAMP processing by both MOR and D3 receptors form the basis for our overall observed hypothesis:

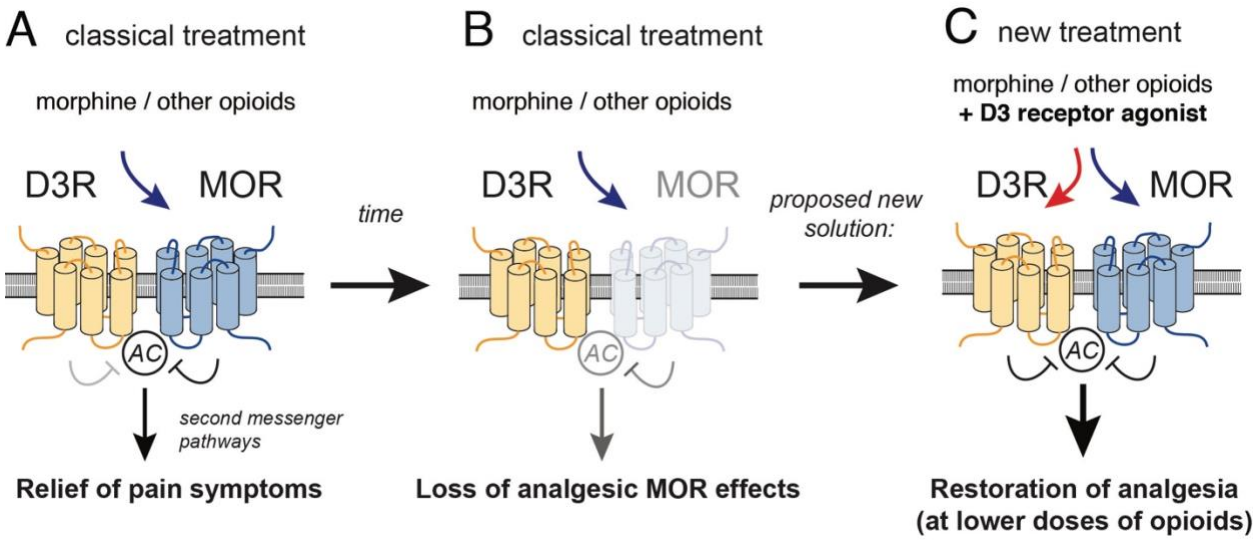

Figure 5: Morphine (5A) action on MOR, and Dopamine (5B) action on D3 pathways leading to the common goal of cAMP inhibition and decreased PKA levels. 5C D3R and MOR work together to inhibit AC production and relief of pain symptoms when used together. Inhibitory actions are increased and opioid levels to achieve pain relief are decreased.

We hypothesize that using MOR and D3 receptor pathways concurrently (Figure 5, panel C), will elicit a synergistic effect on cAMP inhibition. This parallel inhibition in cAMP processing will lead to a more effective and sustained method of pain relief than can be achieved with either of these drugs alone. Second, we hypothesize that this novel combination will achieve analgesia with a lower dose of morphine than if morphine was administered alone. We formulated this hypothesis based on belief that synergistic cAMP inhibition will occur when targeting both MOR and D3 receptor pathways. If we are successful and can achieve analgesia with a lower dose of morphine in combination with D3 receptor modulation, we should be able lower the incidence of negative side effects associated with opioids. This discovery could have a dramatic impact on the field by reducing the risk for tolerance and addiction that is associated with opioid use.

1.10 Previous Studies

Previous studies show that in a centrally induced model of neuropathic pain, a drug combination of morphine (MOR agonist) + pramipexole (D3R agonist) has shown efficacy in achieving analgesia (Rodgers et al. 2020). In this contusion model of spinal cord injury (**SCI**), it was found that only 1/3 of animals responded to morphine after surgery (Rodgers et al. 2021). The remaining 2/3 of animals remained non-responsive, but adjuvant treatment with morphine and pramipexole (**PPX**) was able to provide analgesic responses in all SCI animals. SCI is a massive injury to the CNS and the spinal cord with far-reaching consequences, including paralysis, loss of bladder and bowel function, and the development of neuropathic pain. CNP is highly prevalent in the population of non-SCI patients, but it is unclear if drug treatments that are successful in SCI patients would also be effective in non-SCI patients that present with peripheral chronic pain.

Therefore, the aims to this project are:

Aim 1: Determine the effectiveness of a morphine + PPX combination in a peripherally induced chronic neuropathic pain model via sciatic nerve ligation surgery (**SNL**) in mice.

The analgesic effects of morphine and PPX will be tested separately, then in combination, using an acute treatment protocol. We will induce peripheral neuropathic pain using SNL and test the animal's thermal pain withdrawal latencies as a function of the injury and its responsiveness to drug effects. *We hypothesize that the combination of morphine + PPX will restore withdrawal latencies to match that of the uninjured and contralateral controls. We also hypothesize that morphine alone will have a small analgesic effect on withdrawal reflexes on the SNL injury side, while PPX would have no effect at all. Furthermore, we predict that the morphine dose used in the combination can be reduced by half and still achieve significant pain relief over morphine alone.*

Aim 2: Establish that animals treated chronically with a morphine + PPX combination for peripherally induced chronic neuropathic pain do not develop tolerance to the drug combination.

We will measure withdrawal reflexes in SNL animals over time to assess whether the initially effective combination therapeutic dose remains effective over time. This treatment paradigm reflects the real-world condition where patients undergoing chronic treatment will experience tolerance and a high dose of morphine will be needed to achieve pain relief. *We hypothesize that the morphine/PPX drug combination will maintain its analgesic efficacy over time and animals will show no signs of tolerance.*

Aim 3: Determine the corresponding functional changes in nerve conduction velocities (**NCV**s) and compound action potentials (**CAP**s) of the sciatic nerve in peripherally induced chronic neuropathic pain model and their modulation after morphine + PPX combination treatment.

Preliminary data from our acute drug testing indicates that SNL lowers CAPs and extends NCVs. *We hypothesize that the combination treatment will restore CAPs in SNL nerves to control intensities and restore nerve conduction velocities to control levels than untreated injury.*

CHAPTER 2: Materials and Methods

All experimental procedures were approved by the East Carolina University Institutional Animal Care and Use Committee and were fully compliant with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 80-23). All efforts were made to minimize the number of animals used, while maintaining statistical power.

2.1 Animals:

A total of 35 male C57Bl/6 mice (8-15 weeks old) were used in this study. Animals were housed in the Department of Comparative Medicine space at Brody School of Medicine at East Carolina University. Prior to experiments, animals were randomly assigned into 6 treatment groups.

2.2 Behavioral Testing (Thermal Pain Withdrawal, Hargreaves' Method):

IITX Life Science series 8 model 336 (IITC Inc. Woodland Hills, CA) was used to complete thermal pain withdrawal test as previously described (Cheah, Fawcett and Andrews 2017). In a small room with an air purifier, animals were placed in individual Plexiglas compartments on a raised glass surface. Lights in the room were turned off except for a standing lamp and a desk lamp (light intensity in the room < 100 Lux). Animals were given 30-60 minutes to acclimate to the environment (when there were no injections administered only 30 minutes are given). Under the glass surface a light source was pointed at one hind foot. The light source was armed with the press of a button and a higher, 45% intensity beam was emitted at the second press of the button.

Once the animal has a deliberate foot flick, or licks paw, the button was pressed a third time to turn off the light source and stop the counter. The machine then recorded the time it took between the second and third button press which was then recorded by the experimenter. The intense light beam was set to heat the top of the glass pane to a temperature of 52^{o} C in ~8 seconds. The light system has an automatic 30s shut off point to avoid tissue damage. Each animal had both feet tested 5 times per session, with a 5-minute interval between measurements to avoid adaptation and potential tissue damage.

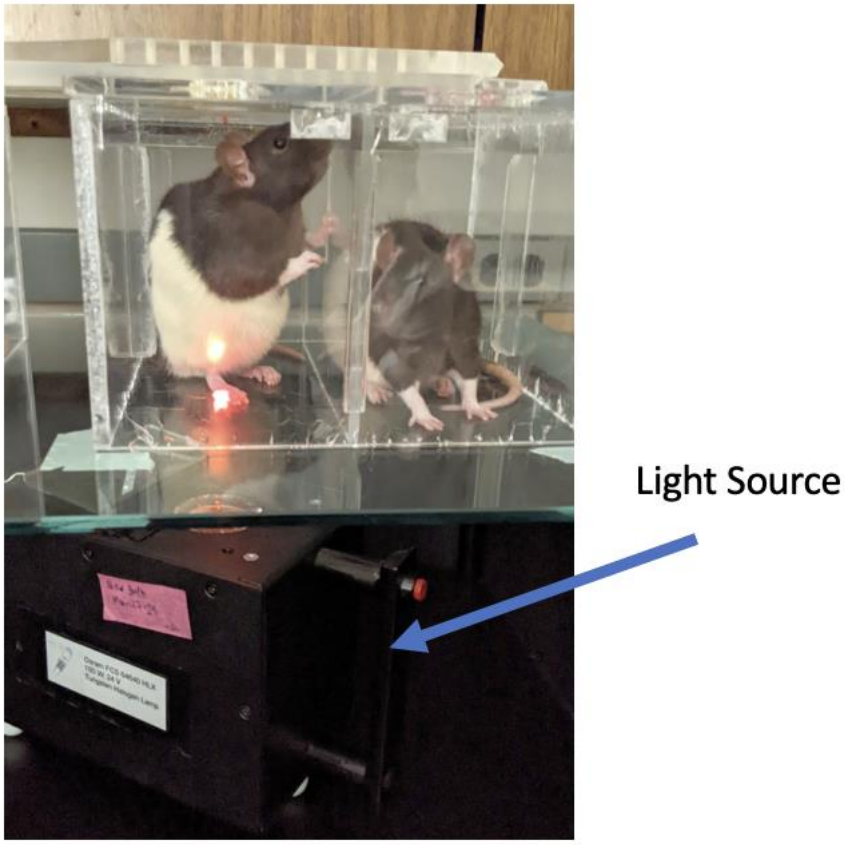

Figure 6: Hargreaves thermal pain withdrawal reflex testing. This test was used to assess pain reflexes of all animals used in this study. The time it takes for them to withdrawal their paw from a high intensity light source indicates pain reflexes. Animals are shown in their individual testing compartments.

2.3 Surgeries:

Chronic constriction of the sciatic nerve was performed as described elsewhere (Bennett and Xie 1988). Prior to surgery, the mouse was anesthetized using 4% inhaled isoflurane, and Nair hair removal cream was applied to the left hind quarters and limb of the mouse. After three minutes, the cream was wiped off along with the hair. Inhaled isoflurane was maintained throughout the procedure at 3-3.5% through a nose cone. The mouse was returned to their normal housing until surgery preparation. The mouse was prepared for surgery under aseptic conditions, with a continuous flow isoflurane apparatus through a nose cone. The mouse was anesthetized with inhaled isoflurane 4% until unresponsive, then maintained at 3-3.5% inhaled isoflurane for the duration of surgery. The mouse was placed in an orientation that holds its nose in the end of a nose cone to administer the isoflurane and taped on the underside of their nose to the cone using gentle paper tape (CVS Health Sensitive Skin Gentle Paper Tape, #336795). Lubricant (CVS Health Nighttime Dry-eye relief NDC#59779-0568-13) was placed on both animal's eyes. The shaved area was treated with alternate applications of betadine and 70% ethanol. While laying on its chest, the mouse was covered with a fenestrated drape exposing the hind limbs and femur areas. The surgeon made a 2 cm incision with a 10-blade scalpel 5 mm below the femur bone on the left side, just through the skin. The muscle layer was exposed, and connective tissue was punctured using fine tipped forceps. The gluteus superficialis and the biceps femoris muscles were then carefully separated to give visualization of the sciatic nerve. The nerve was carefully lifted out from under the muscles and held outside of the animal via forceps placed under the nerve (see figure 7). Sterile saline 0.9% was used to keep the nerve and surrounding tissue lubricated during surgery. With the use of a microscope, a sterile 4-0 braided silk suture was used to tie 3 loose, double knotted ligatures around the animal's sciatic nerve. The loops slid

freely up and down the nerve while slightly touching the nerve all the way around. The double knot ensured that the ligature does not come untied once returned to the animal's body. Once all three ligatures are made, the forceps are removed, and the nerve was carefully tucked back in between the muscle layers. A 4-0 polypropylene suture was used to close the skin layer and 2% lidocaine is applied topically as a post-operative analgesic. The animal was then returned to their normal cage with bedding and observed until they had normal function. Upon resuming normal function in terms of eating and drinking, they were returned to their normal housing facility.

The sham group was subject to the same procedure as the injury groups, but no ligations were made around the sciatic nerve. The nerve was exposed in a similar manner to the injury group and returned to the animal prior to closing the incision and applying lidocaine topically.

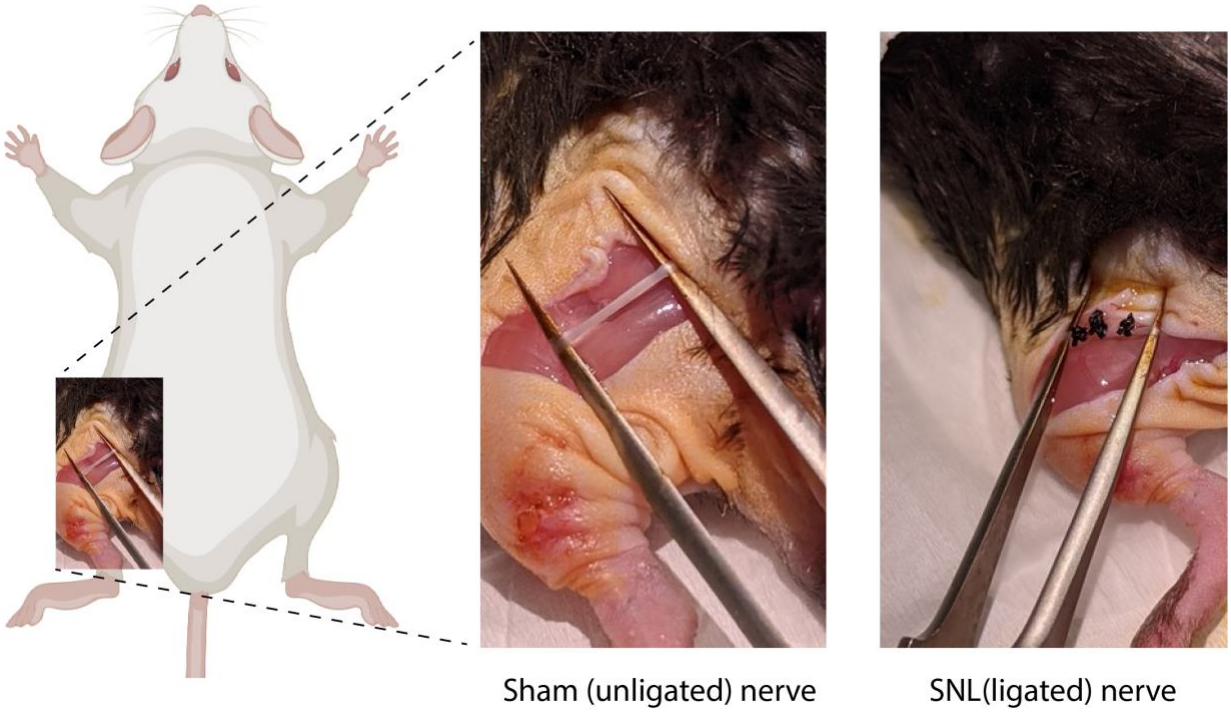

Figure 7: Sham and SNL sciatic nerves. **A.** Exposed sham operated nerve before closing the wound. **B.** Exposed SNL operated nerve with 3 silk ligations before wound closure.

2.4 Pharmacological Treatments in vivo:

Acute drug testing as shown in figure 8. (2-3 days each of morphine 2mg/kg, morphine 1mg/kg, pramipexole 0.5mg/kg, morphine 2mg/kg + pramipexole 0.5mg/kg, and morphine 2mg/kg + pramipexole 0.5mg/kg). The SNL group received the injury, and acute drug testing concurrent with the sham group.

Drug Trials	Dose	Volume
Saline	0.9%	100µL
Pramipexole	0.5 mg/kg	100µL
Morphine	2mg/kg	100µL
Morphine	1mg/kg	100µL
Morphine + Pramipexole	2mg/kg + 0.5mg/kg	100µL + 100µL
Morphine + Pramipexole	1mg/kg + 0.5mg/kg	100µL + 100µL

Figure 8: Animal testing/ treatments for acute treatment protocol. Animals were split into two groups, sham and sciatic nerve ligation (SNL). SNL animals received a chronic constriction injury on their left sciatic nerve. Sham animals received the operation as explained with no ligation injury. Hargreaves testing protocol for both groups is shown here with drug administration parameters. Each drug administration was given to both groups and tested at the same time on the same days.

The remaining 4 groups received the SNL injury, then treatments were performed as shown in figure 9. The saline group received saline i.p. injections of 100 µl every day for 4 weeks, the morphine group received morphine i.p. 1mg/kg/day for 4 weeks, the pramipexole group received pramipexole (PPX) 100µl i.p. 0.5mg/kg/day for 4 weeks, morphine + pramipexole group received i.p. injections of morphine 100µl 1mg/kg + PPX 100µl 0.5mg/kg every day for 4 weeks.

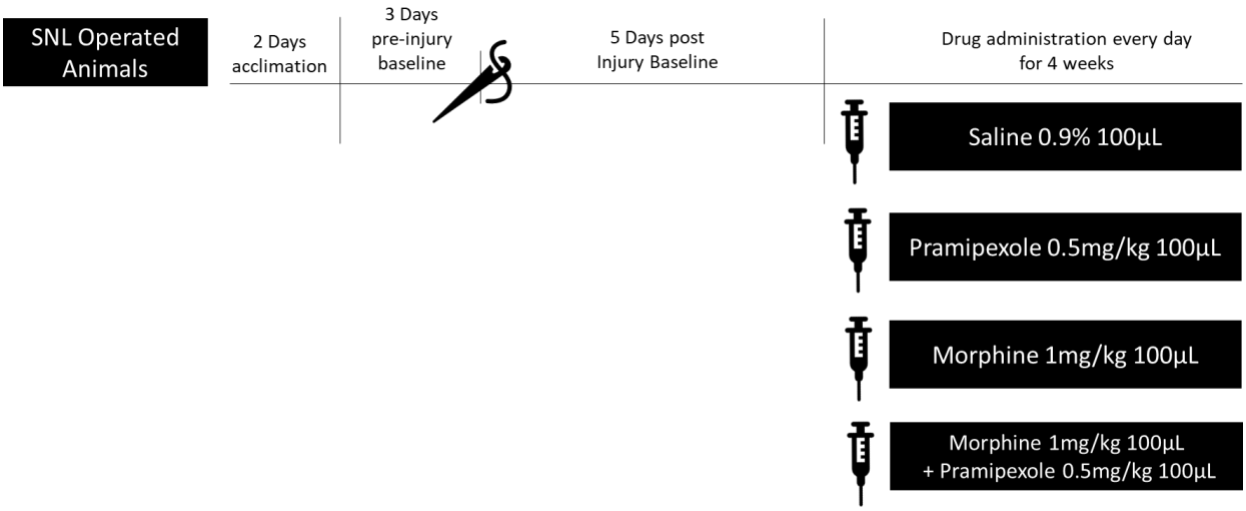

Figure 9: Animal testing/ treatments for the repeated exposure protocol. All animals received SNL injury, and were then split into 4 groups, with 6 animals in each group. Each animal was given the same drug every day for 4 weeks and tested 2-3 times per week. Saline group was tested twice a week and all drug groups tested 3 times a week. All testing occurred at the same time every day.

2.5 Tissue Harvest of sciatic nerves:

Five weeks after surgical intervention, mice were deeply anesthetized using an open drop 100% isoflurane bell jar, and after verification of lack of any reflexes, humanely decapitated. Then, under a microscope (Leica M50), sciatic nerves were carefully dissected from the spinal column to the branching at the knee (Bala et al. 2014) and stored in cooled Locke's Solution (NaCl 154mM, KCl 5.6mM, $CaCl_2$ 2.2mM, Dextrose 5mM, HEPES 2mM in ddH_2O at pH 7.2) for electrophysiological analyses.

2.6 Electrophysiology:

From each animal, control sciatic nerves (contralateral to the ligated nerve) were removed and prepared in a dissection dish filled with cold Locke's solution, and the nerve secured with 10 mm Minutien pins. All connective tissue was carefully removed from the nerve with the aid of the microscope, a probe, fine-tipped eye scissors, and fine-tipped forceps. Electrodes are attached to the nerve at the top and bottom of the preparation, and experimenter ensures a good seal at the opening around the tissue. Once securely attached to the stimulation and recording electrodes, the nerve response is recorded and stored for later off-line analysis. Figure 10 shows the electrophysiological setup and the stimulation and recording configuration.

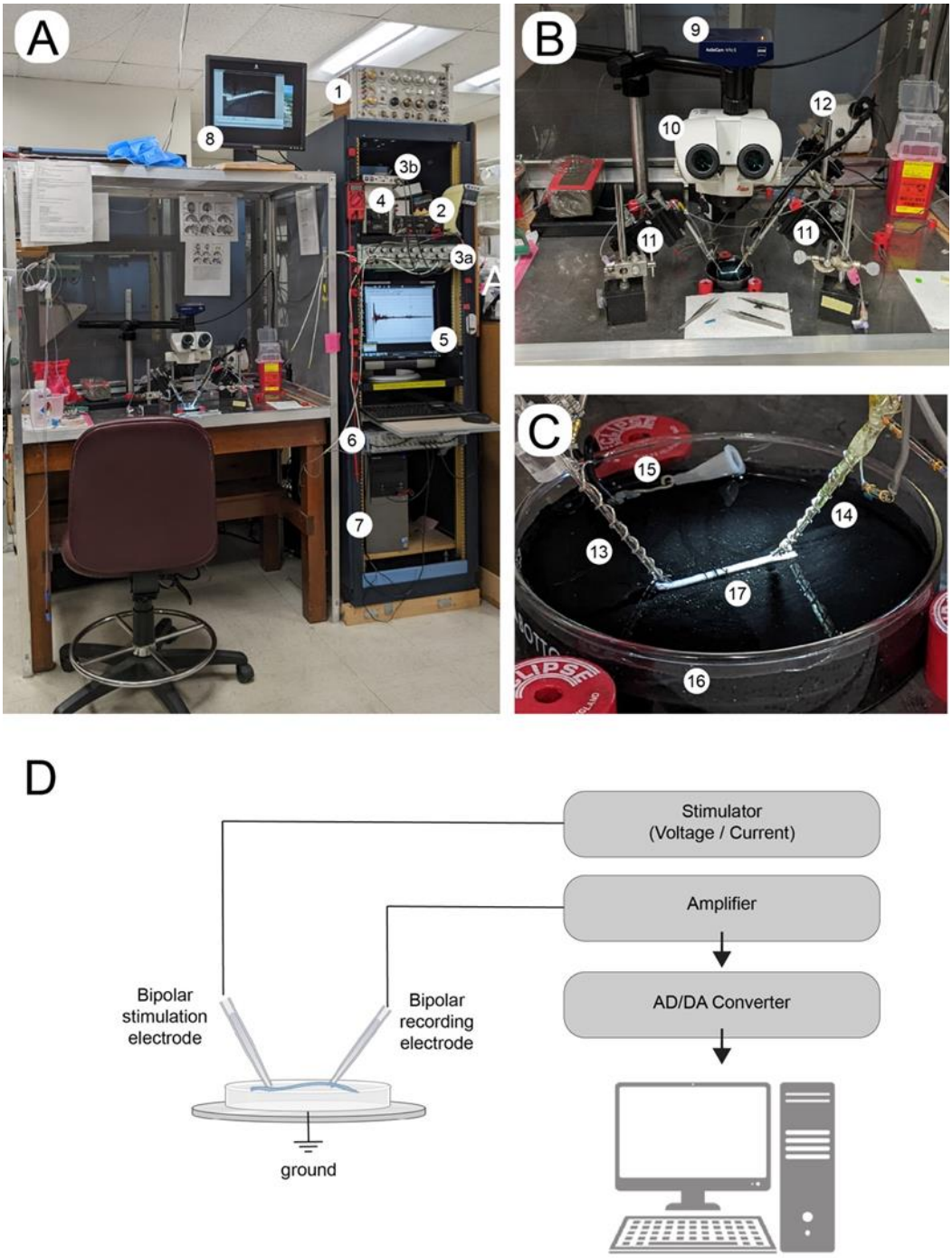

Figure 10: Electrophysiological setup. **A**. Complete setup with Faraday cage. **B.** Positioning of electrodes and micromanipulators around the Petri dish. **C.** Stimulation and recording setup in the Petri dish. **D.** Schematic wiring diagram of the complete setup. 1: Voltage stimulator; 2: Current stimulator; 3a: Differential AC amplifier; 3b: 50Hz/60Hz noise eliminator; 4: light source; 5: monitor; 6: AD/DA signal transformer; 7: PC; 8: video monitor for microscope camera (9); 10: stereo microscope; 11: micromanipulators; 12: light source with gooseneck; 13: bipolar stimulation electrode; 14: bipolar recording electrode; 15: grounding connector; 16: Petri dish with Locke solution; 17: sciatic nerve with ligation.

2.7 Electrode Preparation:

To produce electrodes for electrophysiology experiments, a borosilicate glass tubes with filament (Sutter instruments, #BF150-75-10) is pulled a micropipette puller (Sutter Instruments #P-87). The tips of the individual electrodes are carefully broken off near their tip and then sealed with fire-flaming. Next, the blunted and stabilized tips are carefully sanded on a wet sanding stone (Dan's Whetstone Company, INC, Black AK) until an opening is formed of about 1/4 to 1/3 the diameter of a sciatic mouse nerve. The blunt end of the glass electrode is inserted into a slightly larger diameter piece of tubing, and a small opening is established with the tip of gauge #23 needle, to then insert the internal silver wire. This internal wire has a soldered pin to connect to the appropriate stimulation or recording wiring harness. The wire, tube, and electrode are then secured with epoxy glue. Prior to usage, a second silver wire with a soldered pin is wrapped around the electrode that will serves as the reference electrode in the bipolar stimulation or or recording environment. At the other end of the at approximately 30 cm long tubing, a 16-gauge blunt tip needle is connected to a 3-way Luer connector and secured again with epoxy glue.

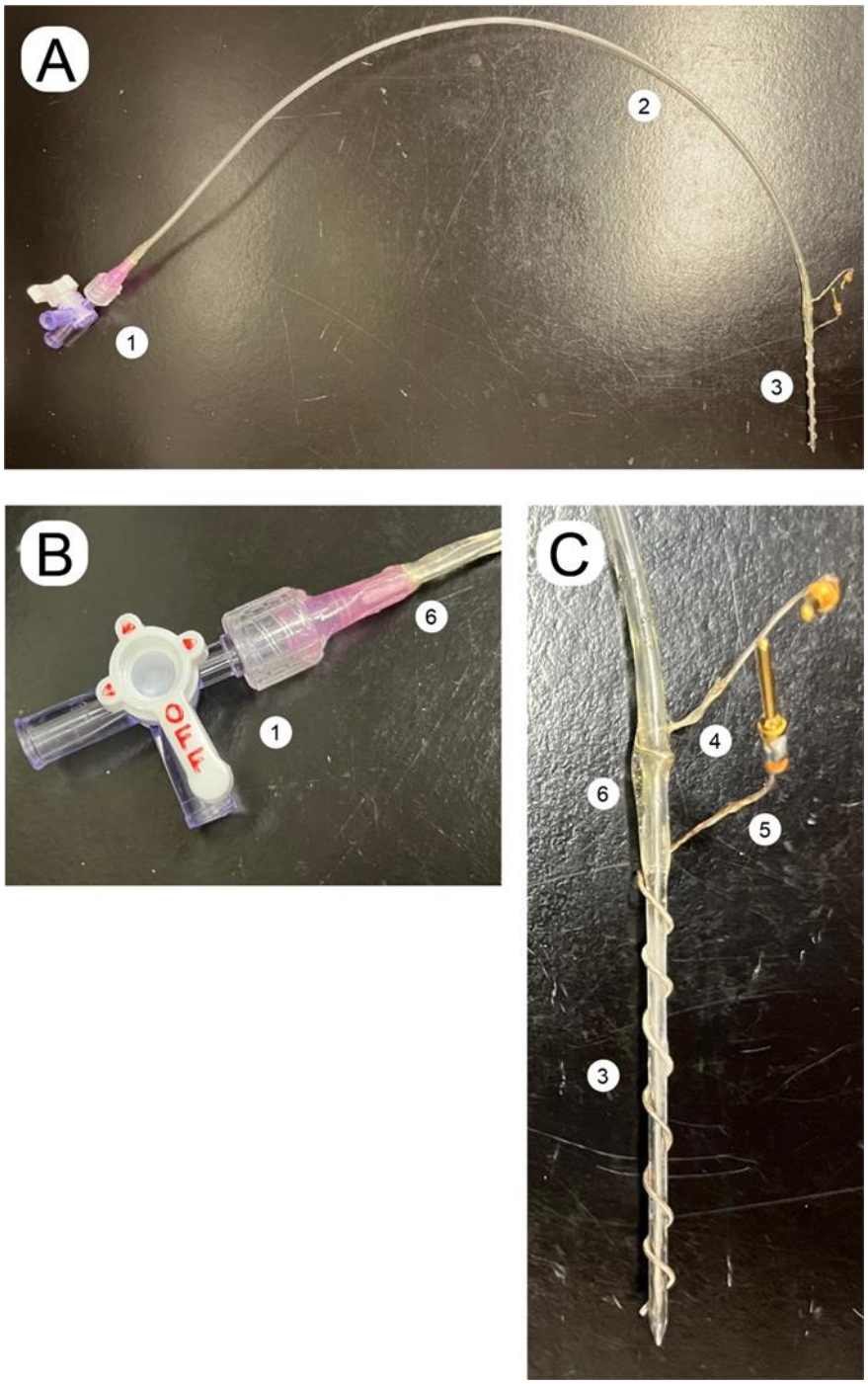

Figure 11: Typical differential electrode with inner (core) and outer (reference) wires. **A.** Overall. **B.** Luer valve end that connects to syringe for suctioning. **C.** Glass electrode with internal and refecence silver wires. 1: Luer connector and lock; 2: polyethylene tubing; 3: glass electrode; 4: internal silver wire; 5: external reference silver wire; 6: epoxy glue.

2.8 Nerve stimulation and recording:

After recovery from dissection and placement, usually ~ 30 min after onset of the experiment, sciatic nerves were stimulated in 15 s intervals under the following parameters:

Intensity	Duration	# of sweeps	Time between sweeps (s)
100 mV	1 ms	30	15
200 mV	0.1 ms	30	15
200 mV	0.5 ms	30	15
50 mV	1 ms	30	15

Signals were recorded with Clampex (Molecular Devices, LLC, San Jose, CA) at 10 kHz, and high- and low-pass filters were set at 100 Hz and 1 KHz, respectively. Signals were stored on a PC and analyzed subsequently with Clampfit (Molecular Devices, LLC, San Jose, CA) and Sigmaplot and SAS (s. below).

2.9 Statistical Analysis:

All experimental data was stored on hard copies, and in Microsoft Excel. Data was then analyzed using Sigma Plot (Version 11, SPSS Science, San Jose, California) and the Statistical Analysis System (SAS). Pre-injury, and post-injury baseline data for the acute experiments were analyzed using a two-way repeated measures ANOVA. Acute pharmacological treatment data was analyzed using a three-way repeated measures ANOVA. In the repeated exposure protocol, the pre-injury, and post injury baseline data was analyzed using a two-way repeated measures ANOVA. The repeated exposure drug testing was analyzed using a three-way repeated measures

ANOVA. When statistical difference was detected, one-way ANOVA, one-way repeated measures ANOVA, T-tests and paired T-tests were used to further analyze data when appropriate. Confidence levels for statistical significance were set at 95% ($p=0.05$).

CHAPTER 3: Results

3.1 Sciatic Nerve Ligation

Sciatic nerve ligation is a moderate form of chronic construction injury that inflicts moderate neuropathic pain and replicates the sensory and anatomical changes that are observed in patients with peripherally induced chronic neuropathic pain (Bennett and Xie 1988, Olaseinde and Owoyele 2022). To generate the murine model of chronic pain, 35 animals underwent surgical intervention (sham control, n=5 or nerve ligation, n=30).

3.2 Baseline Testing

To determine the effect of SNL, we assessed thermal pain withdrawal reflex latencies (**TPWRLs**) on both sides of each animal prior to and after surgery (Figure 12). Sham and SNL surgeries were performed after 5 days of acclimation testing. In animals before sham injury, TPWRLs on either side (black symbols and line: future contralateral control; red symbols and line: future operated sham nerve) ranged from 6-10 seconds and were not statistically different from each other ($p=0.43$, $n=5$). Following the sham surgery, we observed a slight but non-significant decrease in TPWRL on the operated nerve day 1 after surgery ($p=0.18$) that recovered on post-surgery day 2 to the values in the contralateral un-injured side, and that continued to be similar and not significantly different from each other throughout the observed post-surgery period of 10 days ($p=0.79$) (Figure 12, panel A).

SNL animals expressed similar baseline TPWRLs as sham animals that were prior to surgery not significantly different from each other (gold symbols and lines, range: 6-10 seconds, $p=0.39$,

n=6, Figure 12, panel B). In contrast, following SNL, TPWRLs of the injury side dropped from day 1 after surgery to 5.6 +/- 2.1 s and stayed at this range for the remainder of the observed post-surgery period (purple symbols and lines, range: 3.5 to 7 seconds). This difference between injured and non-injured sides was significant across and when compared to sham control values ($p<0.001$). The data stayed significantly different from sham throughout post injury testing and no statistical difference within the SNL group over time was observed ($p=0.42$).

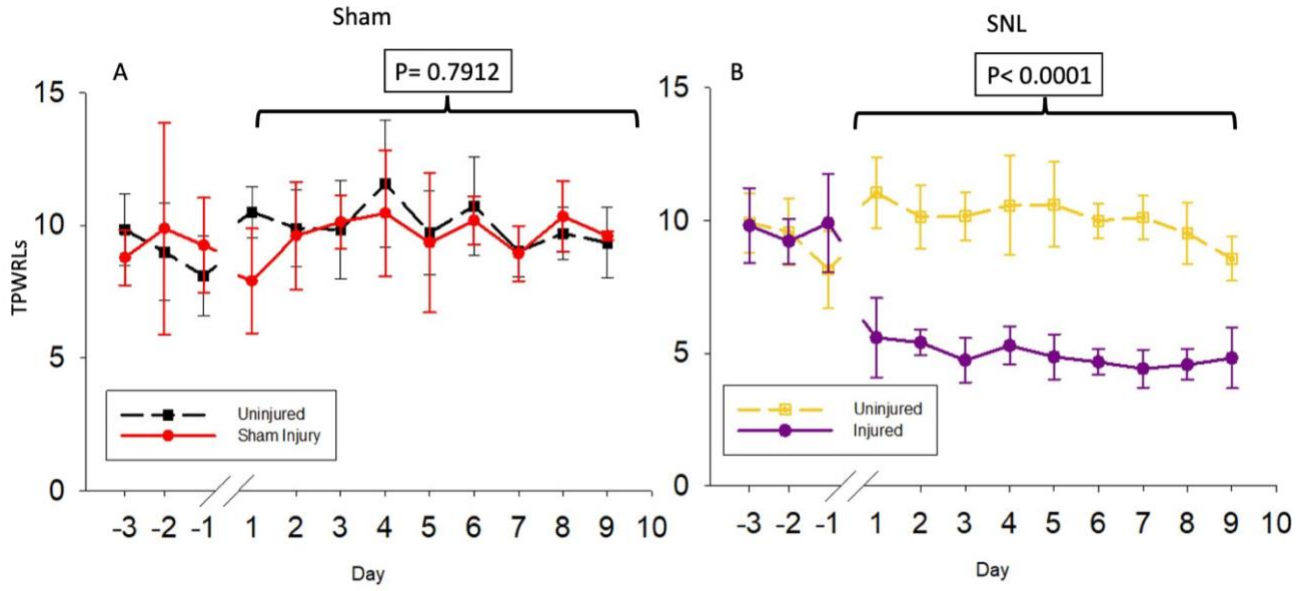

Figure 12: development of TPWRLs over time. **A.** Pre-injury baseline data in the sham animals shows no significant difference between the sham injured and uninjured leg. After the surgery day (indicated by the break in the graph) there was no statistical difference detected with a two-way repeated measures ANOVA. B. TPWRLS of SNL animals. We found no difference prior to surgery day, but TPWRLs were statistically different after surgery for the duration of baseline testing.

3.3 Short-term effects of Pharmacological Treatments

To understand how the SNL model would react to different pharmacological interventions in the short term, we first administered (via i.p. injection) saline (0.9% NaCl, control), PPX (0.5 mg/kg), morphine (at 1mg/kg and 2mg/kg), or either of the morphine doses in conjunction with 0.5 mg/g PPX. The total injection volumes for each drug or combination did not exceed 200 µl per test.

We found that, on the uninjured (sham) side, none of the drugs induced any significant change over pre-or post-injury data ($p = 0.79$) (Fig 13, panel A). Similarly, on the injury (SNL) side, none of the individual drug applications was able to alter reflexes significantly over the post-injury TPWRLs (saline: $p=0.46$; morphine 2mg/kg: $p=0.66$; morphine 1mg/kg $p=0.94$, PPX: $p=0.97$) (Figure 13, panel B). In contrast, both combination treatments (morphine 2 mg/kg +PPX and morphine 1mg/kg +PPX) were able to completely restore TPWRLs to pre-injury levels, showing analgesic effect (morphine 2 mg/kg + PPX: $p=0.54$; morphine 1 mg/kg + PPX: $p=0.34$).

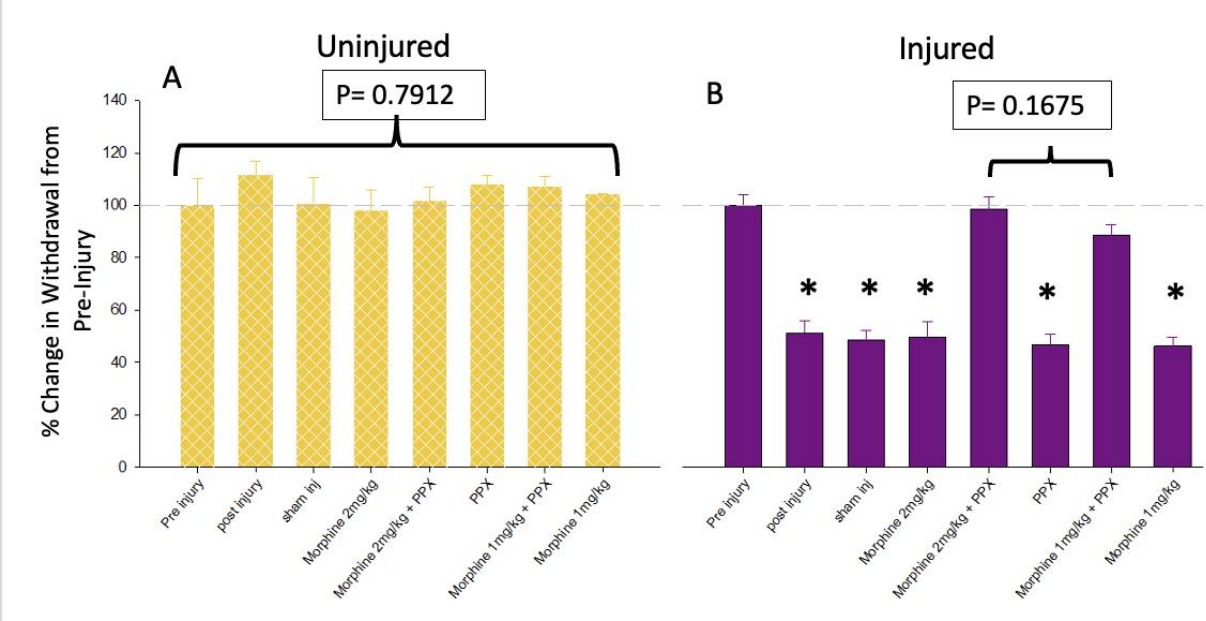

Figure 13: Effects of acute drug treatments. **A.** Averages of withdrawal trials with each treatment condition of right, uninjured leg of the animals that received SNL surgery. No significant difference was detected between any treatment condition. Data is shown normalized to pre-injury withdrawal times of those same legs. **B.** Averages of withdrawal responses of the injured, left side of the SNL injured animals. Data was normalized to pre-injury withdrawal times of those same legs. Post injury, withdrawal times were significantly decreased. No individual treatment led to a recovery of TPWRL, but either administration of the adjuvant treatments did. * indicates significant difference

These data suggest a) that the combination treatment has a synergistic effect in re-stablishing TPWRLs to their normal range that is specific to the side of the injury, and b) that the lower dose of morphine 1 mg/kg can be used to achieve this effect acutely.

3.4 Long-term effects of individual pharmacological treatments

We next probed if the combination treatment with the minimally effective dose of morphine (morphine 1 mg/kg + PPX) would maintain its efficacy over time. All 24 animals in this repeated exposure protocol received the SNL injury and TPWRLs were tested 2-3 times per week depending on group. Animals in the group receiving the sham surgery were injected with sterile saline 0.9% every day for 4 weeks and they were tested bi-weekly. Compared to their contralateral (uninjured) sides (black symbols and lines), TPWRLs on the "injured" side of the sham animal (red symbols and lines) remained significantly reduced at <50% response obtained during baseline ($p<0.001$) (Figure 12, panel A).

In line with the findings after acute treatment (Figure 14), long-term treatment with morphine alone (1 mg/kg/day) did not change TPWRLs on the SNL side (red symbols and lines), and the difference to the un-injured contralateral side (black symbols and lines) remained significant over time ($p<0.001$) (Figure 14, panel B).

Similarly, long-term treatment with PPX alone (0.5 mg/kg/day) did also not change TPWRLs on the SNL side (red symbols and lines), and the difference to the un-injured contralateral side (black symbols and lines) remained significant over time ($p<0.001$) (Figure 14, panel C).

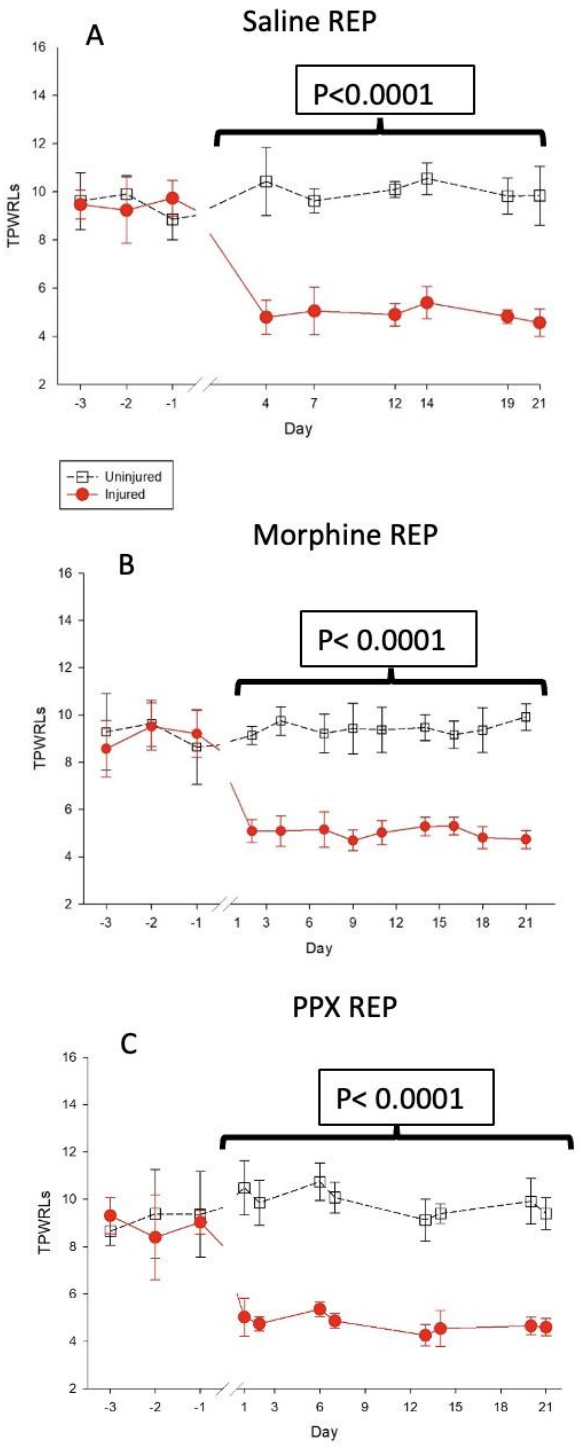

Figure 14: Saline repeated exposure protocol data. **A.** Repeated saline control treatment did not alter TPWRLs over time. **B.** Repeated morphine treatment did not alter TPWRLs over time. **C.** Repeated morphine treatment did not alter TPWRLs over time.

3.5 Long-term effects of adjuvant pharmacological treatment

We next probed if the combination treatment of 1 mg/kg morphine + 0.5 mg/kg PPX, which was effective in the acute setting, maintained its efficacy over time (Fig. 15). We found that the initial acute outcome was preserved over the observation period, and that during the treatment, contralateral un-injured control data (gold symbols and lines) were not significantly different from those of the injured SNL side ($p=0.2$) (Figure 15). These data indicate that the combination treatment retains its analgesic and injury-side specific effect over time.

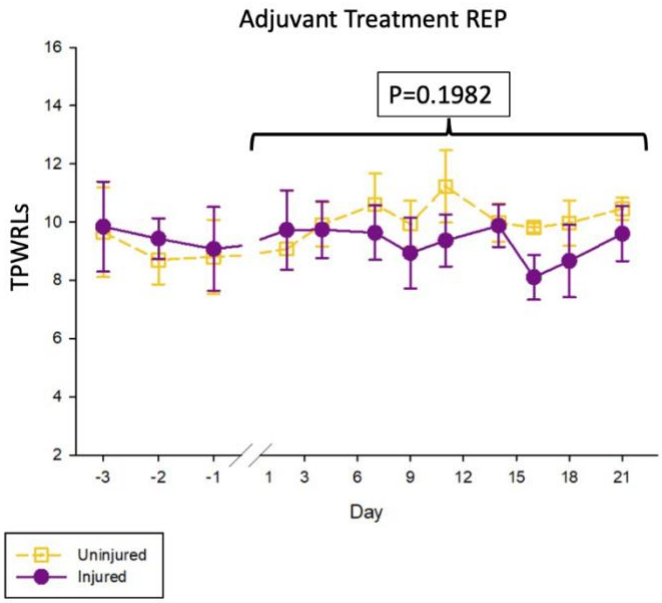

Figure 15: Animal withdrawal responses to adjuvant therapy in repeated exposure protocol. After surgery, animals were treated with morphine 1mg/kg + PPX 0.5mg/kg every day and thermal pain withdrawal reflexes were measured 3 times per week for 3 weeks. Using a two-way RM ANOVA, no statistical difference was detected between the injured and uninjured legs, and there was no change over time.

3.6 Impact of long-term drug treatments on compound action potentials

We next performed an extracellular electrophysiological analysis of compound action potential (CAPs) in SNL and contralateral sciatic nerves to test the impact of the injury and the efficacy of the drug treatments. These studies provide novel data that may provide additional information on the mechanistic underpinnings of the injury-induced changes and the effects of the different drug treatments (Figure 16). We performed recordings from a total of 40 un-injured control and 30 SNL sciatic nerves.

We found that in un-injured contralateral sciatic nerves, electrical stimulation regularly induced a response that consisted of a fast compound action potential (CAP) within ~ 10 ms after the ending of the stimulus (Figure 16, left panels in A, B, C, and D). However, the response on SNL sciatic nerve in the same animal and after saline control treatment was void of the CAP, and only very small electrical events could be typically registered that often extended beyond the typical CAP duration of ~ 10 ms (Figure 16, right panel in A). The effects of long-term treatment with morphine (1 mg/kg/day) were similar to those of after saline treatment (Figure 17, right panel in B). As in saline-treated animals, the responses from the SNL nerves were missing the CAP component but showed some smaller electrical events extending beyond the CAP time frame. Similarly, long-term treatment with PPX (0.5 mg/kg/day) did also not restore CAPs (Figure 17, right panel in C). However, treatment with the morphine-PPX combination that was successful in providing analgesic relief over the duration of the study, partially restored CAPs in the SNL sciatic nerves.

These preliminary data provide the first evidence that the behavioral changes observed could be in part due to underlying restorative events in the injured sciatic nerves. We are currently

performing additional tissue analyses in the hope to better understand this emerging new concept of the combination drug treatment on chronic pain.

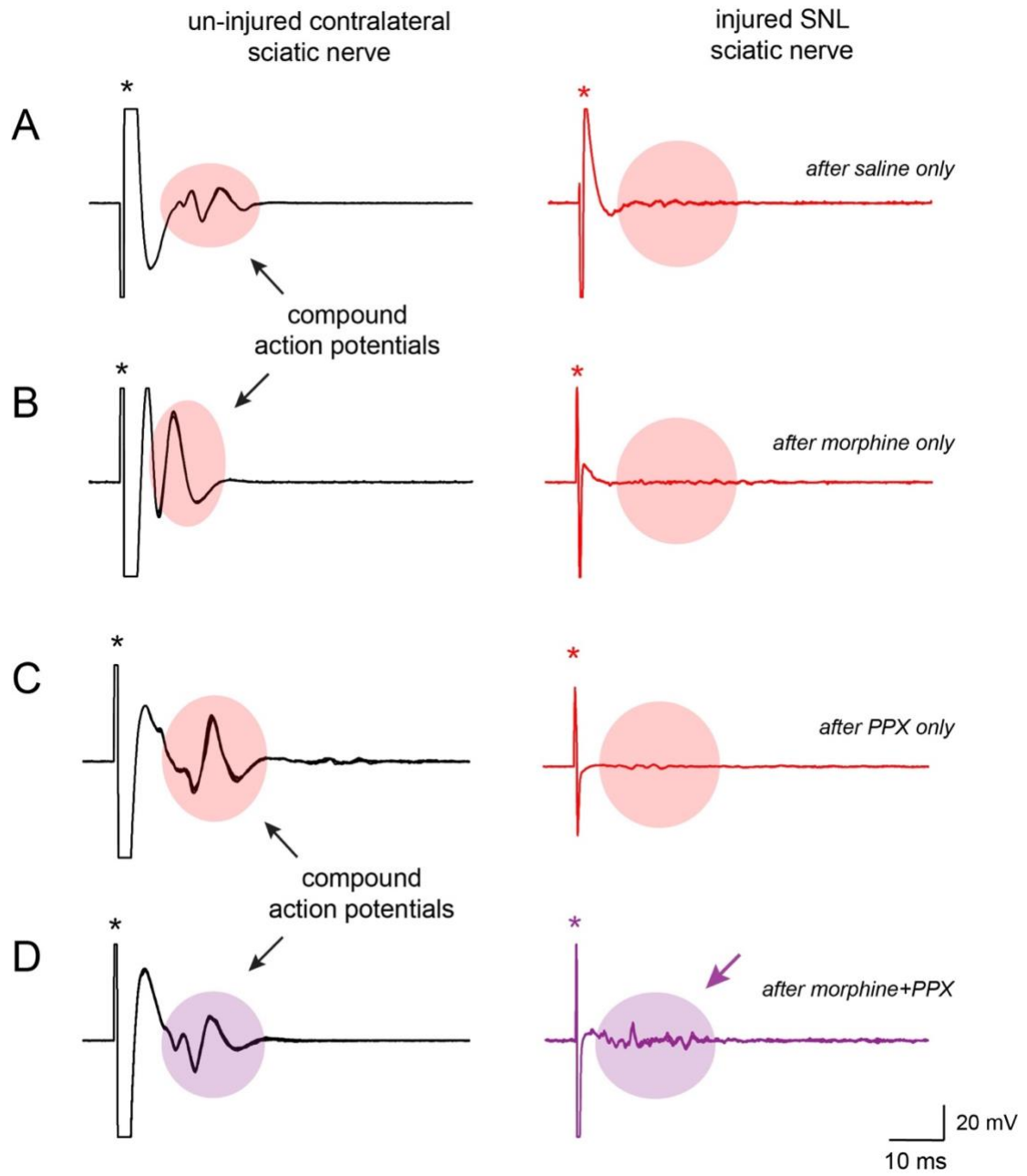

Figure 16: Extracellular electrophysiology recordings of control and SNL sciatic nerves after long-tern drug treatment. **A.** Representative recording of control and SNL nerve after prolonged treatment with saline control. Stimulation of the proximal side of the un-injured sciatic nerve induced a fast response with a single CAP in the intact nerve, but no corresponding CAP was observed in the SNL nerve. Each recording consists of 5 subsequent sweeps that are overlaid. Asterisk (*) denotes stimulus artefact. **B.** Representative recording of control and SNL nerve after prolonged treatment with morphine only (1 mg/kg/day). Stimulation of the proximal side of the un-injured sciatic nerve induced a fast response with a single CAP in the intact nerve, but no corresponding CAP was observed in the SNL nerve. **C.** Representative recording of control and SNL nerve after prolonged treatment with PPX only (0.5 mg/kg/day). Stimulation of the proximal side of the un-injured sciatic nerve induced a fast response with a single CAP in the intact nerve, but no corresponding CAP was observed in the SNL nerve. **D.** Representative recording of control and SNL nerve after prolonged treatment with morphine + PPX. Stimulation of the proximal side of the un-injured sciatic nerve induced a fast response with a single CAP in the intact nerve, and stimulation of the corresponding SNL nerve was able to induce a modest CAP in the injured nerve.

CHAPTER 4: Discussion

4.1 The clinical challenge of CNP

The successful long-term treatment of CNP remains a major clinical challenge. An estimated 10-20 million patients (5-8% of the US population) have chronic pain that restricts their work and quality of life (High-impact chronic pain, HICP (Pitcher et al. 2019, Mackey and Kao 2019, Dahlhamer et al. 2018). As the medical and associated costs with chronic pain exceed $650 billion per year (Mackey and Kao 2019), successfully treating or preventing chronic pain has become an urgent pain management imperative (Skolnick 2018, Mackintosh-Franklin 2018).

CNP is initially responsive to opioids, but long-term opioid treatments generally fail, mainly because of the gradual emergence of tolerance to the prescribed drugs, and the increased risks for dependence and addiction (Ballantyne 2007, O'Connor and Dworkin 2009). Opioids have been readily prescribed to treat pain, and their availability has provided the foundation for the opioid epidemic we are currently facing (Volkow, Benveniste and McLellan 2018). Nearly 5% of the overall adult US population misuses prescription opioids (Skolnick 2018), and there are multiple efforts and policy changes underway to curb this crisis and the availability and prescription practice of opioids in the clinic (Phillips, Ford and Bonnie 2017, Gatchel et al. 2018, Pergolizzi, Rosenblatt and LeQuang 2019, Bonnie, Kesselheim and Clark 2017). Nonetheless, to maintain and accelerate the development of successful pain treatment options, additional approaches are also needed that take advantage of the efficacy of the opioids in treating pain, while avoiding the pitfalls of drug tolerance of addiction.

4.2 SNL as a Model of CNP

CNP can be readily induced in animal models by traumatic brain or spinal cord injury (Kramer et al. 2017, Nicholson 2004). We have shown in earlier studies that spinal cord injury (SCI) is associated with CNP and that dopamine receptor modulators can restore morphine analgesia and prevent opioid preference in this model (Rodgers et al. 2019). We further reported that in this centrally induced model of CNP, these same dopamine receptor modulators can prevent morphine tolerance and reduce opioid withdrawal symptoms (Rodgers et al. 2020). Although the information from the SCI model represented a major discovery in the field, questions about whether a non-SCI model of pain in the context of chronic neuropathic pain would produce a similar outcome were raised. To address this question, we used a sciatic nerve ligation (SNL) model. SNL presents a limited form of chronic construction injury that inflicts moderate neuropathic pain, and replicates the sensory and anatomical changes that are observed in patients with chronic neuropathic pain. We found that SNL induced a CNP phenotype in mice and that the animals did not recover within the observation period of 5 weeks post injury, similar to previous reports in the literature (Delander et al. 1997, Narita et al. 2004). It is well known that central reorganization can occur in chronic pain models resulting from this type of peripheral injury (Goff et al. 1998), and this model facilitates the development of tolerance to morphine analgesia (Christensen and Kayser 2000).

We therefore sought to test if the pharmacological findings observed in the SCI animal model in our earlier work would also be found in the SNL model of CNP. The underlying assumption was that a commonality in pharmacological responses between these two different models of CNP would suggest functional commonalities that might be better targeted in future neuropharmacological studies.

4.3 Effects of acute morphine + PPX treatment after SNL and the establishment of CNP

We had shown earlier that CNP after SCI was generally unresponsive to treatment with morphine or the dopamine D3 receptor agonist PPX, but that we could induce analgesia in morphine-tolerant animals when combining morphine with PPX as an adjuvant (Rodgers et al. 2019). We found that when PPX or morphine were administered on their own, that they did not change TPWRLs indicating that they had no analgesia effect in the SNL injury model if applied alone. In contrast, when used in conjunction, morphine + PPX completely restored thermal pain withdrawal reflex latencies to match that of the contralateral controls. These findings support the notion that the combination treatment of morphine+ PPX can induce an acute analgesic effect, irrespective of the pain model and (likely) the severity of pain state of the animal.

4.4 Effects of long-term morphine + PPX treatment after SNL and the establishment of CNP

As intriguing as the data from the acute treatment outcomes were, we also wanted to test if the analgesic effects persist over time, or if the combination treatment led to the development of tolerance as observed after treatment with morphine alone (Rodgers et al. 2020). Further, a major hallmark of chronic opioid use is the development of tolerance or the need for the dose of opioid to increase to achieve a similar level of pain relief as lower doses. To determine the effects of tolerance in a SNL model we designed a repeated exposure protocol, where animals were treated with saline, morphine, or PPX alone. We found that individual drug treatments showed no effect on TPWRLs over the course of their 4 weeks of testing. In contrast, adjuvant treatment with morphine+ PPX continued to maintain its analgesic effect over time.

Clinically, this could translate to a chronic pain therapy that may not only work better than morphine alone, but also reduces the risk of side effects, tolerance, addiction, and opioid overdose.

4.5 Effects of SNL and drug treatments on CAPs

To better understand the anatomical and physiological changes that occur upon exposure to morphine and PPX, upon completion of the behavioral testing, sciatic nerves were harvested for in vitro extracellular electrophysiology. Nerve recordings were taken at different stimuli to get the cleanest reading and each recording was analyzed using Clampfit software. We found no significant differences in CAPs between sciatic nerves harvest from sham operated sides or their contralateral control. However, in the SNL animals, the size of the CAP of the injured nerves was remarkably decreased, indicating a weaker nerve response on the injured side.

Following the long-term treatments, CAPs from saline, morphine-only, or PPX-only treated animals did not show any sign of change or recovery. However, the adjuvant treatment consistently showed larger CAPS in the SNL-injured nerves than in any of the other groups. While these studies are preliminary and qualitative in nature only, they provide a first indication that the combination drug treatment of morphine+ PPX has the potential to directly modulate changes in the periphery that may underlie, either partially or exclusively, the functional recovery of the TPWRLs in the intact animals.

4.6 Next Steps

Initially, additional goals for this project included an immunohistochemical (IHC) analysis of sciatic nerves and spinal cords, patch clamp electrophysiological analysis on spinal cords, and Western blotting of sciatic nerve tissues to probe for SNL and treatment-related changes in dopamine and morphine receptor protein expressions. We had previously shown that a dysfunction of the μ-opioid receptor (MOR) was associated with changes in dopamine receptor expression (Brewer et al. 2014), while a dysfunction of the dopamine D3 receptor was associated with the upregulation of the dopamine D1 receptor subtype (Yllanes et al. 2015). We have since reported that morphine tolerance can be reversed by blocking D1 or by activating D3 receptor signaling cascades in rodents that had become unresponsive to morphine (Rodgers et al. 2020, Rodgers et al. 2019). As D1 and D3 receptors can form functional heterotetramers with each other (Clemens and Ghorayeb 2019a) as well as with the μ-opioid receptor (Guitart et al. 2019, Ferre et al. 2014, Ferre 2015), and as morphine tolerance involves interactions between the μ-opioid receptor (MOR) and the dopamine (DA) system (Missale et al. 2010, Mazei-Robison et al. 2011, Urs, Daigle and Caron 2011, Zhang et al. 2012, Assar et al. 2016), we are currently analyzing sciatic nerve and spinal cord tissues to probe for SNL and treatment-associated changes that may provide information on the underlying mechanisms that govern the behavioral effects.

In conclusion, our data suggest that both acute chronic systemic administration of the dopamine D3 receptor agonist PPX as an adjuvant to morphine therapy will induce an analgesic response in a peripherally induced morphine-tolerant animal model of CNP. In addition, this adjuvant treatment will maintain its efficacy over time, thus preventing the development of tolerance and

other opioid-associated negative side effects. Taken together, these findings suggest that this drug combination treatment may represent a viable adjunct for long-term morphine therapy to treat chronic pain in the clinic.

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APPENDIX: IACUC APPROVAL LETTER

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | 252-744-2355 fax

January 31, 2022

Stefan Clemens, Ph.D.
Department of Physiology, ECU

Subject: Protocol Q273d, original approval date 09/23/2020

Dear Dr. Clemens:

The amendment#4 to your Animal Use Protocol entitled, "Role of dopamine modulation in the spinal cord." (AUP#Q273d) was reviewed by this institution's Animal Care and Use Committee on 1/31/2022. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

Sue McRae, Ph.D. Chair, Animal Care and Use Committee

SM/GD

enclosure