Behavioral Effects of Opioid Analgesics in the Presence or Absence of Chronic Neuropathic Pain

by

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Dedication

To my parents, who answered some questions when I was a child with “what do the data say about that?”. You taught me to think for myself and exposed me to science early, and you have always been there when graduate school was hard. I really can’t thank you enough for understanding what I’m trying to do and all your support.

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List of Abbreviations

Acc: anterior cingulate cortex

ANOVA: analysis of variance

CCI: Chronic Constriction Injury

CFA: Complete Freund's Adjuvant

D2R: dopamine 2 receptor

DOR: delta opioid receptor

EKC: ethylketazocine

Fixed ratio: FR

GPCR: G protein-coupled receptor

GTPγS: guanosine-5'-O-(3-[^{35}S]thio)triphosphate

i.p.: intraperitoneal

KOR: kappa opioid receptor

Lumbar: L

mg/kg: milligram/kilogram

Mo: months

MOR: mu opioid receptor

NAc: nucleus acumbens

NSAID: non-steroidal anti-inflammatory drug

OUD: opioid use disorder

PET: positron emission topography

PNI: Peripheral Nerve Injury

s.c.: subcutaneous

Sec: seconds

SEM: standard error of the mean

SNI: Spared Nerve Injury

SNL: Spinal Nerve Ligation

THC: Δ^9- tetrahydrocannabinol

TST: Tibial and Sural Transection

Abstract

Chronic neuropathic pain affects ~6.9-10% of the American population. Previous research suggested that chronic pain conditions may increase the susceptibility to developing an opioid use disorder (OUD). Opioid analgesics, or mu-opioid receptor (MOR) agonists, are often used to treat chronic neuropathic pain, such that more than half (~69%) of patients are treated with MOR agonists at some point during their condition. Thus, the goal of these studies was to examine the effect of a neuropathic chronic pain state on the antinociceptive, antihyperalgesic, response rate decreasing, subjective, and reinforcing effects produced by opioid analgesics in both male and female rats. These behavioral effects of the MOR agonists, fentanyl, morphine, and nalbuphine, were evaluated before and after the induction of spared nerve injury (or sham surgery), which produces long-lasting hypersensitivity to noxious stimuli. To evaluate potential pain-relieving effects of MOR agonists, paw withdrawal thresholds were measured in response to increasing mechanical pressure applied to the hind paws. Rates of responding was measured in schedule-controlled responding assays under a fixed ratio (FR)10 schedule of reinforcement. Subjective or discriminative stimulus effects were evaluated in animals trained to discriminate experimenter-administered injections of fentanyl from saline under a fixed ratio (FR) 10 schedule of reinforcement for sucrose pellets. To measure reinforcing effects, animals responded on a FR5 schedule of reinforcement to produce intravenous infusions of fentanyl. Fentanyl, morphine, and nalbuphine produced dose-dependent increases in paw withdrawal thresholds and increases in fentanyl-like discriminative stimulus effects in both male and female rats. Following surgery,

small, but significant, decreases in sensitivity to antinociceptive- and antihyperalgesic-like effects, discriminative stimulus effects, and rate suppressant effects of fentanyl and morphine were observed over time; however, these changes were independent of the presence or absence of nerve injury. While decreases in sensitivity to the rate suppressant effects of nalbuphine were also found in discrimination and schedule controlled responding assays, nalbuphine was more potent and/or effective in producing antinociceptive, antihyperalgesic, and fentanyl-appropriate responding over time. Similar to that observed with fentanyl and morphine, the changes in the effects of nalbuphine were independent of nerve injury. Finally, in self-administration experiments, fentanyl maintained responding in male and female rats. Following surgery, there was a significant decrease in fentanyl self-administration on post-operative day 4 that was resolved/restored by post-operative day 9. Over the remaining 4 weeks of post-surgical evaluation, *independent* of injury, there was an increased intake of specifically the 10 μg/kg dose in both groups. Collectively, these data indicate that chronic neuropathic pain produced few, if any changes, in the behavioral effects of high efficacy MOR agonists, such as fentanyl and morphine. Interestingly, there were changes in the behavioral effects of the partial MOR agonist, nalbuphine, over time that were also independent of nerve injury. The work presented in this dissertation provides novel evidence that chronic neuropathic pain does not alter the behavioral effects of MOR agonists or increase susceptibility to the abuse liability of opioid analgesics. Therefore, increased incidences of OUD diagnoses in chronic pain patients are more likely explained by frequent use of opioids or increased access to opioid prescriptions rather than chronic pain itself.

Chapter 1
General Introduction

1.1 Opioids & Opioid Receptors

1.1.1 Historical Use of Opioid Analgesics

Opioids have been used to treat pain for thousands of years. The earliest reference for use of opioids from the poppy plant in the treatment of pain was in ancient Mesopotamia around 3400 BCE (Norn et al., 2005). In this region, there is some evidence that the poppy plant (Papaver somniferum) was referred to as the "joy plant". Eventually, poppies were grown specifically to harvest opium, and the cultivation of poppies spread via the Silk Road. The Opium Wars began when China (Qing Empire) began to prevent opium imports. Britain wanted to maintain opium trade and used military force to open trade with China during the first and second Opium Wars (Norn et al., 2005).

In 1805, a German chemist, Friedrich Wilhelm Serturner, isolated morphine from the opium poppy (Schmitz, 1985) and subsequent study revealed that morphine produced analgesic effects. Morphine became known as a sort of wonder drug and was used widely. Since opium tinctures varied by batch, pure morphine also improved the ability of physicians to reliably dose patients. Approximately 50 years later, Alexander Wood invented the hypodermic needle and syringe, facilitating the rapid administration of morphine to patients, in particular following injury (Hamilton & Basket, 2000). The end of the Civil War in the United States marked the start of what some consider the first U.S. "opioid epidemic." At this time, opium use in its many forms (e.g., smoking in opium dens, opium tinctures, laudanum) increased. In efforts to find a non-addictive

replacement for morphine, diacetylmorphine (heroin) was synthesized and eventually marketed by Bayer pharmaceuticals as an analgesic and anti-tussive in 1898 (Hosztafi, 2001). However, it was pulled from the market when it became clear that it had strong abuse potential.

At the end of the nineteenth century, reports suggest that more than half of the opioid abuse was reported in upper- or middle-class women who had begun taking morphine or laudanum for general ailments. Rampant opioid misuse eventually led to increased regulation of opioid availability and prescribing practices. In 1914, the Harrison Narcotics Act was passed, which introduced regulations on opioid use such that opioid manufacturers and sellers had to register the drugs they had available as well as pay a small tax (Redford & Powell, 2016; Waterman, 1966). Shortly after the passage of the Harrison Narcotics Act, two cases reached the Supreme Court (United States v. Doremus and Webb et al. v. United States). These cases centered around physicians who were deemed to be overprescribing opioids, and using the verdicts from these cases, the Treasury Department began to attempt to enforce a strict policy against opioid prescribing for maintenance treatment. These regulations made it difficult for individual or independent physicians to prescribe opioids for prevention of withdrawal symptoms. Therefore, about 30 cities around the United States worked to set up opioid clinics to treat opioid-dependent patients. Examples of these cities include New York City, New Orleans, and Shreveport. The goal of these clinics was to provide opioids to patients until abstinence-based treatment could be initiated. However, these clinics were closed by 1920. At this point, opioid-dependent persons had to turn to non-medical sources of opioids to prevent opioid withdrawal symptoms, and drug-related crime increased. It became clear that better treatments for opioid dependence or opioid use disorder were needed. Eventually, methadone, buprenorphine, and naloxone were approved by the FDA for treatment of opioid use disorder (see below).

Following decades of limited opioid prescribing, medical use of opioids for the treatment of pain began to increase in the 1990's. In 1995, at a meeting of the American Pain Society, doctors were urged to consider pain as the "5th vital sign" (Baker et al., 2017). Around the same time, extended-release oxycodone (OxyContin) was released and heavily marketed. These factors likely contributed to increased opioid prescribing, which reached its peak in 2012 with nearly 260 million prescriptions written (Bohnert et al., 2018). Opioid misuse, dependence, and overdose death rates increased as well. In 2017, the United States declared a public health crisis in response to the rising overdose deaths. This is the current opioid epidemic. Since 2017, opioid prescribing has decreased, but opioid overdose deaths still continue to rise.

In sum, clinical use of opioid analgesics (aside from palliative care) for treatment of pain has cycled in and out of favor throughout history. To date, some of the most effective treatments for reducing pain are opioid analgesics, but their clinical use is still complicated due to our inability to separate the useful pain-relieving effects from the euphoric rush or high that underlies the abuse potential of opioid analgesics. Due to abuse potential and the propensity for development of tolerance or physical dependence upon repeated use, opioid analgesics are not typically used today as first-line treatments for chronic pain (Raja et al., 2019). Generally, opioid agonist treatment is a second- or third-line treatment for chronic pain conditions, and opioids are often used in conjunction with other therapeutic agents (Sehgal, Manchikanti, & Smith, 2012; Annemans et al., 2011). For example, in chronic neuropathic pain patients, opioid analgesics are used in approximately 70% of patients, and ~20% of chronic pain patients are maintained on opioid analgesics chronically (Hoffman et., 2017). Therefore, it is important to understand how chronic pain states may alter the behavioral and physiological effects of opioid analgesics.

1.1.2 Opioid Analgesics & Their Receptors

Morphine and other clinically used opioid analgesics bind to and activate the mu opioid receptor (MOR) and induce pain relieving effects as well as constipation, respiratory depression, miosis, nausea, and euphoria. MORs are one of 4 types of opioid receptors: MOR, delta opioid receptors (DOR), kappa opioid receptors (KOR), and the nociceptin/OFQ peptide receptor (NOPR). Opioid receptors are G protein-coupled receptors that couple to intracellular heterotrimeric G protein***s***, consisting of alpha, beta, and gamma subunits. Opioid receptors are coupled to the $G_{i/o}$ G proteins, and agonist binding results in a conformational change in the receptor resulting in dissociation of the G alpha subunit from the beta and gamma subunits (Sharma et al., 1975; North et al., 1987). This dissociation requires the exchange of GDP for GTP and initiates downstream signaling cascades resulting in effects such as inhibition of adenylyl cyclase, but also activation of inwardly rectifying potassium channels and inhibition of both voltage-gated calcium channels (VGCCs) and SNARE protein SNAP-25 (Rhim & Miller, 1***994***, Laugwitz et a***l***., 1993; Hescheler et al., 1987), which ultimately produce antinociception.

1.1.3 Endogenous Opioids

MORs can be activated by exogenous agonists (e.g., morphine) or endogenous opioid peptides. These endogenous opioid peptides can be broadly classified as endorphins, enkephalins, and dynorphins. It was originally thought that endorphins, enkephalins, and dynorphins were selective for MOR, DORs, KORs, respectively; however, recent studies suggest that these ligands may be less selective than originally thought and can bind with high affinity to all opioid receptor types (Gomes et al., 2020).

1.2 Opioid Use Disorder

Opioid use disorder (OUD) is characterized by criteria such as taking drug in larger amounts than intended over time, craving for opioids, persistent desire to cut down despite unsuccessful efforts, and continued use of opioids despite negative consequences or choosing opioid reinforcer over other reinforcers (Wang & Hoyte, 2019). OUD diagnoses include has two additional criteria that are observed with both medical use and illicit misuse, including tolerance following repeated use and withdrawal upon cessation of use. A patient must exhibit 2 or more of the 11 diagnostic criteria to receive an OUD diagnosis, and the number of symptoms determines the severity of the OUD (Boyd & Murray, 2020).

While it is somewhat challenging to estimate prevalence of OUD at a population level, several studies have suggested that OUD diagnoses reached a peak in 2014-2015 with 2.37% of the U.S. population diagnosed with an OUD (Keyes et al., 2022). Recent estimates suggest that currently 6.5-7 million people in the U.S. currently have an OUD (Keyes et al., 2022).

1.2.1 Risk factors for Opioid Use disorder

There are many known risk factors for development of OUD, such as genetic, sex, low socioeconomic status, and mood disorders (Nelson et al., 2013; Kendler et al., 2003; Martins et al., 2012). Chronic pain itself is also considered a risk factor for OUD (Nazarin, Negus, & Martin, 2021). OUD rates are much higher in patient populations diagnosed with chronic pain (~50+%) as compared with the general population (~2% diagnosed) (Juurlink & Dhalla, 2012). Similarly, ~60% of patients with OUD diagnoses experience chronic pain, while only approximately 20% of the general population experiences chronic pain in the U.S. (Cicero et al., 2008; Dahlhamer et al., 2018). Further, ~80% of patients in one study stated that chronic pain was a factor in initial opioid

use (Cicero et al., 2008), and it was also reported that inadequate control of pain increased the probability of misuse of prescription opioids or facilitated use of non-prescription and/or illicit opioids (Bauman et al., 2023). Lastly, in pain patients, more prescriptions or longer-term prescriptions are associated with increased risk of developing OUD (Dowel et al., 2016).

1.2.2 Current Treatments for Opioid Use Disorder

Today, there are three FDA approved treatments for OUD: buprenorphine, methadone, and naltrexone. These treatments are either agonist replacement therapies (buprenorphine, methadone) or designed to prevent MOR agonist-induced effects (naltrexone, naloxone; MOR antagonists).

Methadone

Methadone is a full MOR agonist (Ki ~3.4 nM) (Volpe et al., 2011) and also an antagonist at NMDA receptors (Ki~8.5 nM) (Gorman, Elliot, & Inturrisi, 1997). The extended half-life of methadone allows for once-a-day dosing (Sofuglu et al., 2019).

German scientists developed methadone during World War II as an alternative to opium or morphine, which were in short supply (Joseph, 1994). After the war, the U.S. military brought methadone back to the U.S., and Eli Lily manufactured the compound under the name Dolophine®. The FDA approved methadone as an analgesic in 1947. Several decades of study demonstrated methadone was also effective in decreasing opioid use (Dole, Nyswander, 1965; Dole, Nyswander, Kreek, 1966; Dole Nyswander, 1967; Gearing & Schweitzer, 1974; Larochelle et al., 2017; Sordo et al., 2017), withdrawal, and craving of opioids. This led to FDA approval of methadone as a treatment for OUD (Koehl et al., 2019). Studies demonstrate relatively high rates of patient retention in methadone treatment (Proctor et al., 2015). As a full agonist at MORs, patients do not need to cease opioid use prior to initiation of treatment.

Buprenorphine

Buprenorphine is a high affinity partial agonist at MOR receptors (Ki ~0.2 nM), a KOR antagonist (Ki ~0.11nM), a NOP agonist (Ki=285 nM), and a DOR antagonist (Ki =0.42 nM) (Volpe et al., 2011; Huang et al., 2001). One study has reported buprenorphine to be a DOR agonist (Bidlack et al., 2019), and one of buprenorphine's metabolites, nor-buprenorphine, is a DOR agonist (Huang et al., 2001).

Dr. John Lewis synthesized buprenorphine in 1966 while searching for an opioid analgesic with decreased abuse potential (Heidbreder, Fudala, & Greenwald, 2023). Buprenorphine was FDA approved as an analgesic in 1985 after preclinical and clinical testing and was later approved for treatment of OUD in 2002 (Heidbreder, Fudala, & Greenwald, 2023). Studies demonstrate that buprenorphine is a useful treatment for OUD, with ~40% retention in buprenorphine maintenance treatment programs at the one-year mark (Kennedy et al., 2002). However, as a partial agonist at MOR, it is recommended that patients cease opioid use or be in mild withdrawal prior to beginning treatment to avoid precipitated withdrawal.

MOR antagonists: Naltrexone and Naloxone

Naltrexone is the third FDA approved treatment for OUD and is a MOR antagonist (Ki = 0.3 nM) (Porter et al., 2002). Endo laboratories (now DuPont Pharmaceuticals) created naltrexone in 1963. Naltrexone was eventually FDA approved to treat OUD in 1984, and today, in the treatment of OUD, a depot formulation of naltrexone is used as it has a longer half-life (Srivastava et al., 2023).

While not a treatment for OUD, naloxone is another MOR antagonist, used primarily in the reversal of opioid overdose. Naloxone was developed in the 1960's by Dr.'s Jack Fishman and Mozez Lewenstien. Naloxone was approved to treat opioid overdose in 1971, though use was

mostly confined to hospital emergency settings. The FDA approval of a nasal spray formulation of naloxone in 2017 for over-the-counter use was important for harm reduction efforts, as this increased access to opioid overdose prevention (Ryan et al., 2018).

We Need New Treatments for OUD

While three treatments are FDA approved for treatment of OUD, many patients are without effective treatments or do not seek treatment. Some recent work suggests that only ~18% of patients seek treatment for OUD (Coupet et al., 2021). We need novel, more effective treatments for OUD and non-addictive analgesics. Much ongoing work is focused on further understanding of neurobiological or mechanisms of OUD in order to find novel drug targets for future OUD treatments. Further, current work is also focused on better understanding of risk factors for development of OUD in order to help improve prescribing practices and patient safety. These research efforts are in service of finding novel drug targets for future OUD treatments. Preclinical studies are very useful in the study of underlying mechanisms of OUD and evaluating novel, non-FDA approved treatment.

1.2.3 Preclinical Assessment of Abuse-Related Behaviors

1.2.3.1 Animal Models for Evaluating Reinforcing Effects

To measure drug taking in a laboratory setting, self-administration assays are used (Panillo & Goldbery, 2007). Self-administration assays are generally operant procedures, in which animals learn to make a response that results in delivery of drug (intravenous delivery, vapor, oral consumption), though some oral self-administration studies simply measure total volume of drug consumed. These procedures rely on positive reinforcement, such that if delivery of drug increases the frequency of operant responding, the drug is considered reinforcing (Mackintosh, 1974).

Therefore, self-administration assays can be used in several ways. First, these procedures can be used to determine abuse potential of a drug by measuring drug-maintained responding, such that an animal will self-administer these drugs. Opioid analgesics produce dose-dependent increases responding for drug infusions, suggesting opioid analgesics act as reinforcers (Van Ree et al., 1978). Second, self-administration procedures can be used to evaluate how pretreatment of a drug may alter responding for a primary reinforcer (e.g., drug), which allows experimenters to evaluate potential novel treatments for OUD. A novel OUD treatment should decrease opioid-maintained responding. For example, buprenorphine pretreatment decreases self-administration of MOR agonists (Sorge, et al., 2005).

Classical self-administration experiments (short access) have, however, faced criticism surrounding the temporal pattern of drug use in humans. Consistent with human reports, animals escalate drug intake over time in long-access self-administration procedures (Ahmed & Koob, 1998). Humans are more likely to use drugs in bouts, and this is modeled in intermittent self-administration assays in which periods of drug availability are punctuated with periods during which drug is not available. These models have been more widely used with stimulants (Zimmer, Dobrin, & Roberts, 2011; Kawa, Bentzley, & Robinson, 2016); however, some recent work has examined intermittent self-administration of shorter acting MOR agonists (Bakhti-Suroosh et al., 2021; Fragale et al., 2021).

While intermittent access procedures address temporal patterns of drug use and produce neurophysiological changes observed in human drug users, these procedures still generally use a relatively low and constant work requirement to gain infusions of drug. This is most representative of low effort conditions. Economic theory has been creatively applied to behavioral experiments in order to determine the effort (price) a subject is willing to emit to obtain a given reinforcer as

well as investigate factors related to excessive substance use (Hursh, 1991; Hursh, 1993). To determine the price a subject will pay for a reinforcer, demand curves are generated. In these procedures, 1) price can be systematically increased while dose is held constant or 2) varying the dose of drug, while effort is constant. Both options vary the effort requirement per unit of drug. The slope of a demand curve is used to evaluate the elasticity, or sensitivity to price of a reinforcer. An inelastic demand curve suggests that the subject is insensitive to changes in price such that responding does not drop with increases in price; generally, this is classified as a reinforcer pathology, or evidence of addiction-related behavior as subjects should be sensitive to increases in price/effort (Bickel et al., 2014). MOR agonists are reinforcers (Hursh & Winger, 1995; Ko et al., 2002), and opioid use disorder or opioid dependence predictably alter demand. For instance, it might be expected that a subject in opioid withdrawal would exude more effort to obtain infusions of a MOR agonist than a subject not in opioid withdrawal, reflecting an inelastic demand for opioids in opioid withdrawal. This was indeed seen in morphine dependent monkeys responding for MOR agonist remifentanil; demand was inelastic in opioid withdrawal as compared with non-dependent subjects (Galuska et al., 2007). However, the approved treatment for OUD, buprenorphine, increased elasticity for fentanyl demand (Hammerslag et al., 2020), suggesting buprenorphine increased sensitivity to price/effort increases for fentanyl reward or decreased motivation for fentanyl.

Finally, none of the previously described self-administration experiments capture the element of choice between reinforcers that is present in the human drug taking environment. Assays called "choice procedures" have been developed in which subjects learn that responding on one manipuladum results in delivery of drug, while responding on a different manipulandim results in delivery of a non-drug reinforcer (food reinforcer, access to a social interaction) (Venniro &

Shaham, 2020; Townsend et al., 2019, 2021). Opioid withdrawal increases % opioid choice, for instance (Townsend et al., 2021). One DSM criteria for OUD is choosing opioid reinforcer over other reinforcers, and this can be captured in a choice procedure, unlike in traditional self-administration (single manipulandum) assays. Novel treatments for OUD should decrease opioid choice without largely disrupting behavior allocated to the non-opioid choice (food reinforcer, access to a social interaction).

Lastly, sex differences have been reported in self-administration of opioids. Females often acquire opioid self-administration faster than male rats (Carrol et al., 2002A, B; Lynch & Carrol, 1999; Hu et al., 2004; Jackson et al., 2006); though some studies did not observe a sex difference (Stewart et al., 1996; D'Ottavio et al., 2023). Female rats have been demonstrated to self-administer greater amounts of opioids, though this is most evident at higher work requirements (Carrol et al., 2002; Roberts et al., 1989; Roth et al., 2004).

Self-administration assays are useful ways to learn about drug taking behaviors; however, self-administration assays do not offer information about other aspects of the abuse potential of a drug, such as the drug-induced subjective effects.

1.2.3.2 Preclinical Evaluation of Subjective Effects

Subjective, or interoceptive, effects are known to be related to the abuse potential of a drug (Ator & Griffiths, 2003; Reynolds et al., 2013). Generally, drugs of abuse produce positive subjective effects and, therefore, are readily used and misused. A novel drug that produces similar subjective effects to a known class of drugs of abuse is likely to have abuse potential (Schuster & Johanson, 1988). It is possible to study the interoceptive effects produced by drugs in non-verbal animals through the use of drug discrimination procedures. These procedures have been used in

humans, non-human primates, and rodents. Generally, similar results are obtained across species (Schuster & Johanson, 1988; Riley et al., 2016).

Drug discrimination procedures are extremely useful pharmacological assays as the interoceptive stimuli of a given drug are often drug class specific, and these procedures allow for collection of quantitative in vivo pharmacological parameters. A drug-induced interoceptive stimulus can be trained as a discriminative stimulus through repeated training. In drug discrimination procedures, animals are trained to make injection-appropriate responding following administration of training dose of drug or saline. Following completion of training, training dose and higher should produce responding on the drug-assigned nosepoke, while administration of lower doses of training drug or saline should produce responding on the saline-assigned nosepoke.

Many psychoactive drugs have been shown to produce discriminative stimuli, though this discussion will focus on opioid analgesics. MOR agonist discrimination has been established with many opioids ranging in potency and efficacy (for example, Colpaert & Janssen, 1986; Pournaghash & Riley, 1993; Shannon & Holtzman, 1976; Walker & Young, 1993). Other opioids will generalize to the discriminative stimuli of training dose of a given opioid with expected and predictable differences in potency and efficacy (e.g., partial agonists will occasion less drug-like responding than a full agonist) (Colpaert et al., 1980).

Drug discrimination procedures are extremely useful pharmacological assays as the interoceptive stimuli of a given drug are often drug class specific, and these procedures allow for collection of quantitative in vivo pharmacological parameters. The receptor specificity of discriminative stimuli is exemplified by lack of generalization of other drugs of abuse (Kantak et al., 1999; Bolin et al., 2018) or other opioid receptor agonists to the opioid discriminative stimuli. For example, MOR agonists produce distinct discriminative stimuli from KOR agonists,

suggesting that not all opioid drugs produce similar interceptive stimuli (Negus, Picker & Dykstra, 1990).

These procedures have been used to test the similarity of novel opioids to known opioids, such as morphine as well as to test the ability of potential OUD treatments to decrease the MOR agonist induced discriminative stimuli. Thus, drug discrimination procedures are useful screens for abuse potential of novel drugs in addition to yielding valuable in vitro pharmacological information.

Drug discrimination procedures have also been used to examine the interoceptive stimuli of opioid withdrawal (Holtzman, 2003). These studies involve inducing opioid dependence through daily injections of a MOR agonist and training animals to discriminate between the administration of naltrexone (opioid antagonist) or saline. Naltrexone administration will precipitate withdrawal, while saline injections do not disrupt opioid maintenance. Interestingly, under these stimulus control conditions, non-opioid drugs such as clonidine that are known to alleviate some components of opioid withdrawal produce responding on the saline-assigned nosepoke rather than the naltrexone-assigned nosepoke (Holtzman, 2003). This extension of the drug discrimination procedure suggests that subjects can differentiate changes in physiological state.

Other work has examined how exposure to painful stimuli may alter the interoceptive stimuli of opioids in both humans and rodents. Several studies have examined the subjective effects of other opioids in the presence or absence of exposure to a noxious stimulus in subjects with no opioid-dependence and opioid-dependent subjects. In non-opioid dependent subjects, several studies demonstrate that the pleasurable or euphoric subjective effects of codeine or fentanyl were decreased when human subjects were exposed to noxious stimuli such as 2°C water (Conley et al.,

1997; Comer et al., 2008; Zacny & Beckham, 2004). One study included an opioid-dependent and a non-opioid-dependent group and the findings suggest that in non-opioid dependent subjects, opioids were rated as relatively unpleasant, while opioid-dependent subjects and hospitalized patients (most of the group experiencing chronic pain but not all) reported pleasurable effects from opioids (Lasagna et al., 1955).

Further, several pre-clinical studies have examined how exposure to relatively short acting pain states may alter the discriminative stimuli of opioids. Neonatal exposure to visceral pain failed to alter the morphine discrimination dose effect curve in adulthood (Norwood et al., 2014). Injections of 0.4% acetic acid (i.p.) produced a ~2.2-fold shift in the morphine dose effect curve in males while acetic acid injections did not alter the oxycodone dose effect curve in males or either dose effect curve in females (Neelakanten et al., 2015). The authors attributed the lack of acetic acid induced shift in the morphine or oxycodone dose effect curve in females to greater antinociceptive potency of oxycodone in females (Neelakanten et al., 2015).

An interesting study that did not include an opioid tested how chronic pain would alter the discrimination of a drug with a weak discriminative stimulus, namely, aspirin (Wiessman et al., 1976). Arthritic animals were more accurate in their discrimination of aspirin from saline, though the sham animals also successfully learned discrimination of aspirin-saline. This study suggests that pain relief may be a discriminable state.

Sex differences have been reported in the subjective effects of opioids in humans. Several studies have shown that men report greater levels of pleasurable subjective effects of opioids and lower levels of unpleasant subjective effects while women experience the opposite (Comer et al., 2010, Zacny, 2001). In rodent discrimination studies, opioid analgesics tend to be more potent in

female rodents than males (Craft et al., 1996), though we know relatively little about sex differences in opioid discriminative stimuli as few studies include female rodents.

1.3 Pain

1.3.1 Types of Pain

Pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" by the International Association for the Study of Pain (IASP) (Raja et al., 2019). Further classification can distinguish reflexive nociception (e.g., removing a hand from a hot burner) from inflammatory pain (e.g., post-operative pain, some types of arthritis) caused by tissue damage, swelling, and abnormal levels of inflammatory markers. Neuropathic pain can originate from diseases, such as diabetes or herpes, or from injury (spinal cord injury; herniated disc, etc). When painful sensation persists past the healing period of the injury, or lasts for longer than three months, pain is considered chronic. Chronic pain is a large public health problem today, with 20-25% of U.S. population experiencing chronic pain (Dahlhamer et al., 2018). Unfortunately, the prevalence of pain is expected to continue to rise as pain-associated diseases continues to rise (e.g., diabetes, cardiovascular disease, cancer, etc).

While the sensory component of pain is generally considered to describe the intensity or magnitude of pain sensation (Horn et al., 2012), the affective component describes the unpleasantness of the pain (Talbot et al., 2019). The sensory component or intensity of the painful stimuli is correlated with the unpleasantness. The affective component of pain may last longer than the actual injury and may coincide with feelings of fear or depression, if the pain experience persists, such as in chronic pain, and may coincide (Talbot et al., 2019). Some studies have

suggested that experimental pain or pain of a known origin is generally rated as less unpleasant than clinical or potentially unknown pain (Dunckley et al., 2005; Strigo et al., 2002).

While pain is generally regarded as an unpleasant experience, ability to experience pain is considered an evolutionary advantageous trait; experiencing pain alerts individuals to potential or actual tissue damage or injury (Bonavita & De Simone, 2011). The utility of pain sensation is perhaps best exhibited by the marked reduction in life expectancy and increase in likelihood of injury in individuals with a congenital inability to feel pain (Congenital Insensitivity to Pain and Anhydrosis (CIPA) (Daneshjou et al., 2012; Schon et al., 2020).

1.3.2 Nociceptors & Pain Circuitry

Exposure to painful stimuli activates nociceptors; these are a class of receptors that recognize pressure, temperature, or chemical stimuli (Dubin & Patapoutain, 2010). Activation of these receptors results in depolarization of primary afferent neurons, and the action potential is propagated along Aδ or C fibers. The signal can be redirected into a spinal reflex loop (nociception), or/and it can continue to spinal regions involved in pain such as the dorsal root ganglion (DRG) to the dorsal horn of the spinal cord; this is referred to as the ascending limb of the pain pathway. The descending limb of the pain pathway begins with supraspinal regions sending signals to the dorsal horn of the spinal cord, and the encoded information results in behaviors aimed to decrease further injury such as favoring an injured limb or decreased levels of movement (Bannister, 2019; Shah & Hanauer, 2007).

1.3.3 Pre-clinical pain states

There are many pre-clinical pain states, and these can be broadly categorized as examples of nociceptive-, inflammatory-, or neuropathic-like pain states. Generally, nociceptive-like pain states are escapable and short acting and tend to be shorter term pain, encompassing pain originating from skin or tissue damage (somatic) as well as visceral or abdominal pain. However, inflammatory- or neuropathic-like pain states are inescapable and longer-lasting. These models are often used to investigate novel treatments for pain or to learn about the physiological changes induced by pain. While injury can induce inflammation, several substances also produce inflammation and have been used to examine antinociceptive-like or antihyperalgesic-like effects in animals, including carrageenan (Winter et al., 1962), formalin (Abbot et al., 1995), complete Fruend's adjuvant (Fruend, Casals, & Hosmer, 1937), and heat killed mycobacterium to induce an arthritic like state (Canang & Pearson, 1978).

Neuropathic pain can be somatic (related to the body), visceral (internal, non-specific location), or idiopathic (unknown) in origin and is caused by disease- or trauma-induced damage to nerves. There are a few neuropathic pain states designed to mimic disease-related neuropathic pain. Animals exposed to a high fat, high sugar diet, and then given repeated injections of streptozotocin develop a hypersensitive state, similar to that observed in diabetic neuropathy (Davidson et al., 2014; Yorek et al., 2016 for review). Chemotherapeutic induced neuropathic pain is also a problem clinically, and animals given repeated injections of paclitaxel, vincristine, cisplatin, or other chemotherapeutic agents develop a hypersensitivity to mechanical stimuli (Hoke & Ray, 2014). There are many neuropathic pain states involving damage to the sciatic nerve or spinal cord during surgery. Surgical injuries to the sciatic nerve are commonly used in preclinical research because the sciatic nerve is large and relatively easy to identify, isolate, and manipulate. The spinal nerve ligation (SNL) and peripheral nerve injury (PNI) models involve isolating and

damaging lumbar (L)4-6 vertebrae. The SNL model involves isolation and ligation of the L5 and L6 nerves (Kim &Chung, 1992), while the PNI consists of isolating and damaging the L4 nerve (Masuda et al., 2017). Several neuropathic pain states involve damage to specific branches of the sciatic nerve such as the tibial and sural transection (TST). This surgery involves removing a 2 mm section of the tibial and sural nerve branches while sparing the peroneal (Lee et al., 2000). The chronic constriction injury (CCI) involves constricting all three branches of the sciatic nerve with suture (Bennet & Xie, 1998). Last, the spared nerve injury (SNI) model involves removing a 2 mm section of the tibial and peroneal nerves while the sural nerve is left untouched (Decosterd & Woolf, 2000).

All of these pain states induce hypersensitivity to noxious stimuli, but the duration of the induced hypersensitivity differs. The SNI model has been shown to last for at least 8 months (Erichsen & Blackburn-Munro; Decosterd & Woolf, 2000), indicating a very persistent hypersensitive state, enabling experimenters to ask longitudinal questions. The SNI model was chosen for the experiments described in this thesis due to the highly persistent state of hypersensitivity.

1.3.4 Pre-clinical Assessment of Pain-related Behaviors

In humans and animals, painful states increase or decrease the rate or frequency of some behaviors (pain-elicited or pain-depressed behaviors, respectively). Behaviors that increase in the presence of pain are called nocifensive behaviors (Stevenson et al., 2006). These behaviors are typically observed in humans following exposure to noxious stimuli, e.g., reflexive retraction of a hand from a hot burner or pan. Common examples of pain-elicited behavior in animals include the retraction of a limb or tail from a noxious stimulus, such as lifting an injured paw in the hot plate or retracting an injured hindpaw from applied mechanical pressure in an experiment such as Von

Frey or Randall Selitto (Randall, 1957). These assays are similar and both measure paw withdrawal thresholds, though the Randall Selitto technique has the distinct advantage of not requiring the animal to bare weight on the injured paw (Randall 1957). An analgesic drug should result in a decreased reaction or a delay in the reaction to the noxious stimuli in pain-elicited behavior assays. Therefore, any drug-induced sedative effects or general suppression of locomotion could be interpreted as an antinociceptive-like effect (Stevenson et al., 2014; Negus et al., 2006, Morgan et al., 2008).

Patients tend to seek medical attention when pain decreases normal function or activity, such as feeding, locomotion, or engaging in normal, daily tasks (Martin et al., 2004; Morgan et al., 2008). This is referred to as pain-depressed behavior. Pain-depressed behaviors can be evaluated using operant and non-operant assays. Examples of pain-depressed behavior in rodents include decreases in wheel running, food intake, or food-maintained responding in an operant paradigm (Morgan et al., 2008; Martin et al., 2004). The advantage to these experimental methods is that rate suppressant effects of drugs will further decrease behavior, preventing a false-positive antinociceptive response. Both types of assays have experimental benefits, and the best approach may be to combine these assays.

Many previous studies have demonstrated that MOR agonists produce antinociceptive- or antihyperalgesic-like effects in both pain-elicited and pain-depressed behavioral assays. In a pain-elicited assay, this is observed as a decreased sensitivity to the noxious stimulus or increased latency to nocifensive behavior. In a pain-depressed behavioral assay, antinociceptive- or antihyperalgesic-like effects would be observed by restoration of pain depressed behaviors.

Sex differences have been previously reported in a both baseline pain thresholds in some studies (Li et al., 2009). In the SNI model specifically, few studies have used females, but one

study demonstrated lower paw withdrawal thresholds in female rats compared with male rats (Ahlstrom et al., 2021). Some studies have also shown that there are sex differences in the potency of opioids to alleviate pain, such that opioids are less potent in female rodents (for example: Barrett, Smith, & Picker, 2002; Cook et al., 2000; Cook & Nickerson, 2005; Craft et al., 2001; Peckham & Traynor 2005, 2006; Terner et al., 2002; 2005; for review: Craft et al., 2003). In humans, generally, women are more sensitive to painful stimuli (Walker & Carmody, 1998), and women are more likely to experience some forms of chronic pain (Bouhassira et al., 2008; Colloca et al., 2017). Opioid analgesics are also less potent in women, similar to that observed in rodents (Cicero et al., 1996; Craft et al., 2008, for review of rodent, human similarities).

1.3.5 Reinforcing Effects of Opioid Analgesics in Pain States

Several human studies have examined how chronic pain alters the reinforcing effects of MOR agonists. These studies examined if chronic pain altered oxycodone-money choice in human subjects. Participants made responses to obtain either money or oxycodone, and the work requirement varied. In subjects with chronic pain, significantly more responses were made for oxycodone than money. However, in pain-free subjects, more responses were made for money than oxycodone, suggesting that oxycodone was less reinforcing in pain-free subjects (Comer et al., 2008) or that money was more reinforcing in pain-free subjects. Further, oxycodone did not maintain responding in pain-free, opioid naïve subjects, unless they were exposed to experimental pain (cold pressor task), suggesting pain states may increase the pleasurable effects of oxycodone (Comer et al., 2010; Walsh et al., 2008). These results may collectively suggest that previous history of opioid use and pain exposure alter the abuse potential of oxycodone and other opioid analgesics.

Previous preclinical studies have examined the impact of a pain state on self-administration of MOR agonists. Generally, many of these studies show decreased acquisition of MOR agonist self-administration in the presence of a pain state (Martin et al., 2007; Kupers & Gybel, 1995; Lyness et al., 1989; Woller et al., 2014; Wade et al., 2003). These findings have been interpreted as a decrease in or decreased sensitivity to the reinforcing effects of MOR agonists. Two studies evaluated if pain states (CFA (inflammatory), lactic acid, capsaicin (nociceptive)) would alter ongoing self-administration of opioid analgesics, and these studies found no change in opioid-maintained responding (Barattini et al., 2023; Reiner et al., 2021) While previous studies have many methodological differences, one commonality is use of acute or sub-chronic pain states. This limits the available time to evaluate behavior, and many previous studies evaluated the impact of pain in between-subject designs.

Therefore, the goal of the present study was to determine if a long-lasting, chronic neuropathic pain state alters self-administration of fentanyl. This fills several gaps in previous literature. First, use of a chronic pain state allows us to repeatedly examine behavioral effects of opioid analgesics in the presence or absence of chronic pain in a within-subject design. Second, previous studies using neuropathic pain states in rats have only evaluated if neuropathic pain states alter the acquisition of opioid analgesic self-administration (Lyness et al., 1989; Kupers & Gybel, 1995; Woller et al., 2014; Wade et al., 2003) in rats. These studies have generally reported a decrease in the abuse potential of opioid analgesics. One study reported that in mice trained to self-administer morphine, paclitaxel induced chemotherapeutic neuropathy produced a small, but significant increase in morphine breakpoint in male mice (but not female mice) (Neelakanten et al, 2017). Therefore, in this study, we evaluated MOR agonist-induced behaviors prior to induction of chronic neuropathic pain and then repeatedly, within-subject, re-evaluated the effects of sham

or SNI states on MOR agonist induced behaviors. In the present study, we evaluated the fentanyl discriminative stimulus and fentanyl self-administration prior to and after induction of chronic neuropathic pain; however, in the future, it is important to consider if neuropathic pain states will alter other portions of the cycle of addiction (see Fig. 1-1; adapted from Venniro et al., 2020). Third, as previous work has primarily focused on male rodents, we included both male and female rodents in all studies. One previous study evaluating both males and females observed increased self-administration of fentanyl in the presence of CFA-induced pain states in only male rats (Higginbotham et al., 2002), while another study reported no sex differences in fentanyl intake in presence or absence of CFA-induced pain states in rats (Barattini et al., 2023). Both of these studies induced pain states prior to acquisition. Only one study in rats included males and females and trained animals to self-administer opioids prior to the induction of pain (lactic acid, capsaicin); this study reported no sex differences and presented the data collapsed across sex (Reiner et al., 2021).

1.3.6 Subjective Effects of Opioid Analgesics in Pain States

Previous work has examined how exposure to painful stimuli may alter the interoceptive stimuli of opioids in both humans and rodents. Several studies have examined the subjective effects of other opioids in the presence or absence of exposure to a noxious stimulus in subjects with no opioid-dependence and opioid-dependent subjects. In non-opioid dependent subjects, several studies demonstrate that the pleasurable or euphoric subjective effects of codeine or fentanyl were decreased when human subjects were exposed to noxious stimuli such as 2°C water (Conley et al., 1997; Comer et al., 2008; Zacny & Beckham, 2004). One study included an opioid-dependent and a non-opioid-dependent group and the findings suggest that in non-opioid dependent subjects, opioids were rated as relatively unpleasant, while opioid-dependent subjects and hospitalized

patients (most of the group experiencing chronic pain but not all) reported pleasurable effects from opioids (Lasagna et al., 1955).

Further, several pre-clinical studies have examined how exposure to relatively short acting pain states may alter the discriminative stimuli of opioids. Neonatal exposure to visceral pain failed to alter the morphine discrimination dose effect curve in adulthood (Norwood et al., 2014). Injections of 0.4% acetic acid (i.p.) produced a ~2.2-fold shift in the morphine dose effect curve in males while acetic acid injections did not alter the oxycodone dose effect curve in males or either dose effect curve in females (Neelakanten et al., 2015). The authors attributed the lack of acetic acid induced shift in the morphine or oxycodone dose effect curve in females to greater antinociceptive potency of oxycodone in females (Neelakanten et al., 2015).

An interesting study that did not include an opioid tested how chronic pain would alter the discrimination of a drug with a weak discriminative stimulus, namely, aspirin (Wiessman et al., 1976). Arthritic animals were more accurate in their discrimination of aspirin from saline, though the sham animals also successfully learned discrimination of aspirin-saline. This study suggests that pain relief may be a discriminable state.

Overall, these previous drug discrimination studies hint that the subjective effects of opioids may differ in the presence of transient pain state, but no studies have examined the effects of a long-lasting pain state on the discriminative stimuli of opioids. The preclinical assays described above are well suited for pre-clinical evaluation of if chronic pain states alter the abuse potential of opioid analgesics.

1.4 Chronic Pain Induced Changes in the Opioidergic System

In human and rodent studies, changes in the opioid receptor system have been observed in pain states. Decreased expression of MORs was observed in patients with various types of chronic

pain (fibromyalgia, migraine, back pain, etc) (Harris et al., 2007; DosSantos et al., 2012; Lamusuo et al., 2017; Martikainen et al., 2013; Schrepf et al., 2016). In animal studies, neuropathic pain states also generally resulted in decreased MOR expression in the spinal cord, DRG, insula, caudate putamen, and motor cortex compared with controls (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Pol et al., 2006; Porecca et al., 1998; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998). However, several studies have demonstrated no change in MOR expression in inflammatory pain states in rodents (Delay-Govet et al., 1989; Milan et al., 1987). These results suggest that the type of pain state may differentially alter MOR expression, but that decreased MOR expression is expected in neuropathic pain states. Since MOR expression is decreased in many pain states, it might be expected that pain should decrease sensitivity to MOR agonist-induced behavioral effects; however, it would be useful to further study brain region specific changes in expression.

Changes in endogenous opioid peptide expression have also been observed during pain states in rodent and human studies. There is some evidence that chronic pain patients have lower levels of circulating beta-endorphin (Bruehl et al., 2012, 2013, 2014; Rhodin et al., 2013). In the streptozoticin model of diabetic neuropathy, diabetic rats had elevated levels of met-enkephalin peptide in the spinal cord (Kolta et al., 1996). However, there was no difference in plasma met-enkephalin levels in diabetic patients with or without neuropathy (Fallucca et al., 1996). Several studies have demonstrated an increase in levels of met-enkephalin, beta endorphin, or dynorphin in areas such as the striatum, frontal cortex, and spinal cord in animals exposed to inflammatory pain states (Zangen et al., 1998; Cesselin et al., 1980). It is possible that increased expression of endogenous opioid peptides could promote desensitization and downregulation of opioid

receptors, including MORs. Therefore, we would predict that pain decreases sensitivity to the behavioral effects of MOR agonists.

1.5 Dopamine Receptor System & Chronic Pain Induced Changes

Previous studies also demonstrated changes in the dopaminergic system in pain states in humans and rodents. Dopamine D2 receptors (D2R) have been most widely studied in the context of various types of chronic pain and human imaging and rodent studies found a decreased expression of D2 receptors in chronic pain as well as an overall lower level of dopamine (released) (Hagelberg, et al., 2003; Martikainen et al., 2015; Sagheddu et al., 2015; Wood et al., 2007). Fibromyalgia patients had decreased levels of [^{11}C] raclopride (D2R) binding compared to healthy controls when exposed to a noxious stimulus, which could suggest increased release of dopamine in fibromyalgia patients. Increased release of endogenous dopamine could promote desensitization of D2Rs. Alternatively, it is possible that reduced levels of D2Rs could promote greater release of dopamine as D2Rs are autoreceptors (Wood et al., 2007).

Decreased D2R expression in the nucleus accumbens (NAc) and anterior cingulate cortex (Acc) was observed in neuropathic pain in rats (Hakim et al., 2020; Ortega-Legaspi et al., 2011; Selley et al., 2020). Formalin treatment decreased D2R expression, D2R agonist induced G-protein activation, and D2R downstream signaling in the NAc core (not shell) (Pzaki et al., 2002). D1R and D2R expression and levels of tyrosine hydroxylase (rate-limiting step in dopamine synthesis) were decreased 2-4 weeks after SNI surgery (Sagheddu et al., 2015). Collectively, these changes would predict decreased sensitivity to dopaminergic drugs in a pain state. Several previous studies evaluated drug-evoked dopaminergic release in the presence or absence of a pain state. Generally, pain states decreased the evoked dopamine following systemic or intra-VTA administration of

opioids and cocaine but not amphetamine (Hipolito et al., 2015; Ozaki et al., 2002; Taylor et al., 2015).

1.6 Experimental Objectives

The overall goal of this dissertation was to determine if chronic pain altered sensitivity to MOR agonist-induced behaviors over time. Since previous studies report that MOR expression is decreased in chronic pain, we hypothesized that MOR agonists would be less potent following chronic nerve injury as compared with non-injured rats. Further, we hypothesized that these shifts would be observed to a greater extent in female rats than male rats. Generally, previous literature has suggested decreased sensitivity to MOR agonist induced antinociceptive-like effects in female rodents compared with male rodents (for example: Barrett, Smith, & Picker, 2002; Cook et al., 2000; Cook & Nickerson, 2005; Craft et al., 2001; Peckham & Traynor 2005, 2006; Terner et al., 2002; 2005; for review: Craft et al., 2003), and female rats have a smaller receptor reserve than male rats (Peckham & Traynor, 2005). This hypothesis was evaluated in three chapters. Understanding if chronic pain states increase the abuse potential of MOR agonists could help inform future treatment of chronic pain and treatment of patients with both chronic pain and opioid use disorder.

1.6.1 Chapter 2: The effects of chronic neuropathic pain states on MOR-agonist induced antihyperalgesic-like effects and rate suppressant effects

1.6.1.1 <u>Study 1:</u> The effects of chronic neuropathic pain states on sensitivity to MOR agonist-induced antihyperalgesic- or antinociceptive-like effects in male and female rats

Opioid analgesics have been well documented to produce antihyperalgesic- and antinociceptive-like effects; however, little is known about how ongoing pain states may alter

sensitivity to MOR agonist induced antinociceptive- or antihyperalgesic-like effects. The first study in this dissertation aimed to determine if MOR agonist-induced antihyperalgesic- or antinociceptive-like effects were altered over 6 months of ongoing, persistent hypersensitivity. MOR agonists ranging in potency and efficacy were selected (fentanyl, morphine, and nalbuphine) as well as non-opioidergic drugs previously shown to attenuate hypersensitivity (gabapentin [calcium channel blocker], diclofenac [non-steroidal anti-inflammatory agent], Δ9-tetrahydrocannabinol (THC) [cannabinoid receptor partial agonist], ethylketocyclazocine (EKC) [kappa opioid receptor agonist], SNC80 [delta opioid receptor agonist], cocaine [dopamine reuptake inhibitor], quinpirole [non-selective dopamine D2, D3 agonist]). Drug induced alterations in mechanical hypersensitivity were measured using the Randall Selitto assay. MOR agonists were evaluated before and after induction of pain, and other drugs were evaluated after. All experiments utilized both male and female rats.

Overall, fentanyl, morphine, and nalbuphine induced dose-dependent antihyperalgesic- and antinociceptive-like effects in both male and female rats. There were small, but statistically significant, leftward shifts in the fentanyl and morphine dose effect curves over time observed in rats with spared nerve injury and non-injured controls. Unexpectedly, nalbuphine was significantly more potent at later post-operative timepoints in both sham and SNI groups, suggesting SNI-induced hypersensitivity did not induce this change in sensitivity. Together, these data indicate that chronic pain did not alter the opioid-induced antihyperalgesic- and antinociceptive-like effects. However, changes in the potency of MOR agonists occurred over the course of this study, suggesting that other factors such as repeated exposure to drug or age may be responsible for the increased sensitivity to nalbuphine-induced antinociceptive- or antihyperalgesic-like effects as

well as small decreased sensitivity to fentanyl- or morphine-induced antihyperalgesic- and antinociceptive-like effects.

1.6.1.2 <u>Study 2:</u> The effects of chronic neuropathic pain states on sensitivity to MOR agonist-induced rate suppressant effects.

While it has been well documented that MOR agonists induce rate suppressant effects, we do not know if a painful state may alter the sensitivity to the effects of MOR agonists. Further, it is important to concurrently examine rate suppressant effects when interpreting a pain-elicited behavioral assay (Randall Selitto). Therefore, a separate group of animals was trained to respond for sugar pellets under a fixed ratio (FR)5 schedule of reinforcement in a multi-component schedule maintained responding assay. Rate decreasing effects of MOR agonists and non-opioidergic compounds were evaluated prior to surgery and over 6 months of ongoing neuropathic pain states.

Overall, fentanyl, morphine, and nalbuphine dose-dependently decreased rates of responding prior to surgery. Following either sham *or* SNI surgery, small, but statistically significant, rightward shifts in the potency were observed. Four to six months after either sham *or* SNI surgery, nalbuphine was significantly less rate suppressant, such that large doses of nalbuphine did not decrease rates of responding. These observed changes are not explained by presence of chronic neuropathic pain; therefore, it is possible other factors such as repeated exposure to drug or age may underly the decreased sensitivity to opioid induced rate suppressant effects over time.

1.6.2 Chapter 3: The effects of chronic neuropathic pain-like states on the reinforcing effects of a highly potent MOR agonist, fentanyl

Several previous studies have examined how opioid self-administration is altered by presence of a pain state. Many of these studies have induced a pain state prior to acquisition of self-administration of MOR agonists. These studies have, for the most part, found a decrease in acquisition of MOR agonists in a pain state (Martin et al., 2007; Kupers & Gybel, 1995; Lyness et al, 1989; Wade et al., 2003; Woller et al., 2014). Only two previous studies have demonstrated that induction of an acute or sub-chronic pain state did not alter ongoing self-administration (Barattini et al., 2023; Reiner et al., 2021). Overall, the previous studies have almost exclusively used acute or sub-chronic pain states. Use of a chronic pain state would provide several experimental advantages. First, a longer lasting pain state increases the time available to evaluate opioid analgesic-induced behaviors and allows examination of possible pain-induced changes over time. Second, no previous studies have examined if a chronic neuropathic pain state alters ongoing self-administration of drugs of abuse. Therefore, the goal of the present study was to determine if SNI altered the reinforcing effects of highly potent MOR agonist, fentanyl, or the indirect dopaminergic agonist, cocaine.

In these experiments, fentanyl maintained-responding was significantly decreased on post-operative day 4 (first day resuming self-administration post-operatively) in both sham and SNI groups. By post-operative day 9, self-administration of fentanyl was similar to pre-surgical levels. Over the 4-week post-operative period, fentanyl self-administration was subject to small, but statistically significant changes, though the observed escalation of intake of 10 ug/kg/inf fentanyl were observed in both sham and SNI groups. This suggests that SNI-induced hypersensitivity did not alter fentanyl intake, though there were small, significant increases in fentanyl intake over time *independent* of pain states. Other factors such as repeated exposure to drug likely explains the

increased self-administration of high dose (10 μg/kg) fentanyl and is consistent with previous studies (Dao et al., 2021; Malone et al., 2021).

Cocaine self-administration was not significantly altered over the 4-week postoperative period in either sham or SNI groups. Collectively, these findings suggest that SNI-induced hypersensitivity fails to alter the reinforcing effects of fentanyl *or* cocaine. This study was started with the intention to consider sex as a biological variable; however, all data are presented as a mixture of males and females due to catheter failures in female rats.

1.6.3 Chapter 4: The effects of chronic neuropathic pain states on the discriminative stimulus effect of fentanyl and other MOR agonists

Subjective effects are known to contribute to the abuse potential of drugs (Ator & Griffiths, 2003; Reynolds et al., 2006). In humans, several previous studies found that exposure to painful stimuli decreased the pleasurable subjective effects evoked by prescription opioids, such as oxycodone (Comer et al., 2010; Zacny & Beckman, 2004). Though, we do not yet understand whether or not chronic neuropathic pain state may alter the discriminative stimulus of MOR agonists.

To assess how ongoing chronic pain states may alter the discriminative stimulus of MOR agonists, rats were trained to discriminate 0.032 mg/kg fentanyl from saline. Prior to sham or SNI surgery, discrimination training and pre-surgical testing of other compounds were completed. Following sham or SNI surgery, fentanyl, morphine, and nalbuphine dose effect curves were repeatedly evaluated over 4 months. A separate group of animals was trained to discriminate 5.6 mg/kg cocaine from saline.

Following either sham or SNI surgery, small, but statistically significant, rightward shifts were observed in fentanyl and morphine dose effect curves. After sham or SNI surgery, small

doses of nalbuphine produced higher levels of fentanyl-appropriate responding, suggesting an increase in sensitivity to nalbuphine-induced interoceptive effects. Collectively, SNI failed to alter the discriminative stimulus of fentanyl, though changes in sensitivity to opioid analgesic induced interoceptive effects were observed over time.

1.6.4 Conclusions

In the current studies , changes were observed in sensitivity to behavioral effects of opioid analgesics over time. Small, but significant, decreases in sensitivity to behavioral effects of fentanyl and morphine were observed over time. Increased sensitivity to nalbuphine induced antinociceptive-like effects, antihyperalgesic-like effects, and the discriminative stimulus of nalbuphine was observed; however, decreased sensitivity to the nalbuphine-induced rate suppressant effects was observed. Possible explanations for these time-based changes in sensitivity to behavioral effects of opioids are discussed.

Previous work has suggested that chronic pain is a risk factor for OUD. Therefore, the experiments in this dissertation sought to determine if chronic nerve injury altered sensitivity to the behavioral effects of MOR agonists. Overall, the data presented in the following chapters suggests that chronic nerve injury failed to alter sensitivity to behavioral effects of MOR agonists. These studies suggest that chronic nerve injury did not directly alter the reinforcing, subjective, or analgesic-like effects of MOR agonists. These findings were in contrast to our initial hypothesis that chronic pain states would decrease the sensitivity to MOR agonist-induced effects. While chronic nerve injury itself did not alter the reinforcing effects of MOR agonists, there may be other explanations for why chronic pain is a risk factor for OUD. For example, in chronic pain, patients are often prescribed opioid analgesics and, therefore, have increased access to medically prescribed MOR agonists. Additionally, patients may be taking opioid analgesics frequently and/or

repeatedly, which would lead to tolerance and the use of larger doses. Therefore, it is possible that repeated exposure to drug or increased access to drug during treatment for chronic pain may underlie pain-related risk factors for OUD.

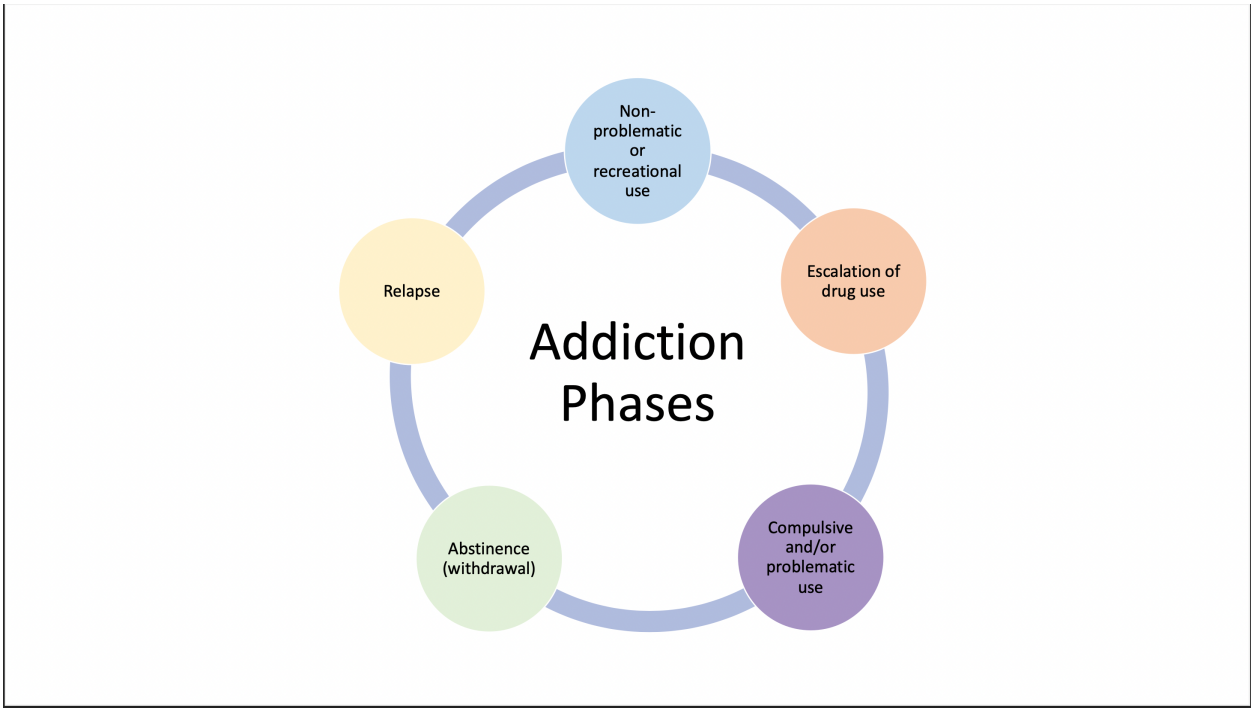

Figure 1-1. Phases of the addiction cycle (adapted from Venniro et al., 2020)

Depiction of phases of the addiction cycle

Chapter 2
The Effects of Chronic Neuropathic Pain States on MOR Agonist-Induced Antihyperalgesic-like Effects and Rate Suppressant Effects

2.1 Abstract

Chronic neuropathic pain affects 6.9-10% of people in the United States. While mu opioid receptor (MOR) agonists are not used as first line treatments for chronic pain, recent data suggest that~70% of chronic neuropathic pain patients were maintained on MOR agonist treatment at some point. Several previous studies demonstrated decreased MOR expression and activity in pain states as compared with non-injured or sham controls; however, we know relatively little about how chronic pain states may alter the antinociceptive- and antihyperalgesic-like effects of MOR agonists in vivo. To determine if MOR agonist induced antinociceptive- and antihyperalgesic-like effects were altered in the presence of spared nerve injury (SNI) induced hypersensitivity, fentanyl, morphine, and nalbuphine were administered prior to surgery and repeatedly following surgery. MOR agonist induced antinociceptive- and antihyperalgesic-like effects were evaluated in SNI and sham groups repeatedly over a 6-month following surgery. MOR agonists induced dose-dependent antinociceptive- and antihyperalgesic-like effects in both male and female rats with the expected rank order of potency (fentanyl>morphine≥nalbuphine). Over time, there were small rightward shifts in fentanyl and morphine induced antinociceptive- and antihyperalgesic-like effects; however, these rightward shifts were observed in both sham and SNI groups, suggesting this occurred independent of pain state. Interestingly, nalbuphine was more potent 6 months following surgery than 3 months in both sham and SNI groups. This suggests the potency of

nalbuphine was changed over time but was not altered by SNI. Other, non-MOR agonist analgesics were also evaluated. Collectively, these data indicate that SNI failed to alter the antinociceptive- and antihyperalgesic-like effects of MOR agonists, though changes over time were observed in the potency of nalbuphine independent of pain state. Future studies should evaluate if the observed increase in sensitivity to nalbuphine is related to the partial KOR agonist activity of nalbuphine and if SNI-induced hypersensitivity alters the development of tolerance to the antinociceptive- and antihyperalgesic-like effects of MOR agonists.

2.2 Introduction

Chronic neuropathic pain affects 6.9-10% of Americans (Scholz, et al., 2019) and is defined as pain originating from an injury or disease state affecting the somatosensory nervous system by the International Association for the Study of Pain (IASP) (Raja et al., 2019). Examples of chronic neuropathic pain include diabetic neuropathy, chemotherapeutic induced neuropathy, or post-stroke pain (Scholz et al., 2019; Rice, Smith, & Blyth, 2016; van Hecke et al., 2014).

First-line treatments for neuropathic pain include antidepressants (e.g., tricyclic antidepressants or serotonin and norepinephrine reuptake inhibitors) and anticonvulsants (pregabalin, gabapentin) (O'Connor et al., 2009; for recent review, see Bernetti et al., 2020). Many neuropathic pain patients report that these first-line treatments do not produce satisfactory relief from symptoms (Dworkin et al., 2007; Scholz et al., 2019). Therefore, second- and third-line treatments for neuropathic pain are used frequently and include topical lidocaine patches and mu opioid receptor (MOR) agonists (Cavilla et al., 2019; O'Connor et al., 2009). While there has been some debate surrounding the effectiveness of MOR agonists in treating neuropathic pain, numerous studies report MOR agonists produce analgesia in neuropathic pain states across many

species, including in humans (Joubert et al., 2020). Further, some studies demonstrated MOR agonist treatment resulted in higher levels of patient satisfaction than other treatments (Widerstrom-Noga & Turk, 2003). Therefore, it is perhaps not surprising that MOR agonists are used in many chronic neuropathic pain patients with approximately 69% of patients receiving MOR agonists at some point and ~20% of patients maintained on MOR agonists chronically (Hoffman et al., 2017). Treating chronic pain requires prolonged periods of repeated dosing with MOR agonists, so there is generally always some concern about development of tolerance to analgesic effects or misuse (O'Connor et al., 2009). It is well documented that repeated use of MOR agonists can produce tolerance to MOR agonist induced effects, requiring dose escalation to produce the same effects (Ballantyne and LaForge, 2007; Walker & Young, 2001). Using large doses of MOR agonists is linked to increased risk of developing an opioid use disorder (OUD) as well as risk of overdose (Ballantyne &LaForge, 2007; Hayes et al., 2020; Henry et al., 2014; Morosco et al., 2015).

Although MOR agonists are frequently used in chronic neuropathic pain treatment, we understand relatively little about how chronic pain may alter the effects of MOR agonists. For example, MOR expression levels and MOR-induced G protein activation are decreased in chronic pain states (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Pol et al., 2006; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998). Additionally, studies demonstrate decoupling of the MOR from the G protein in brain areas involved in reward and pain perception in chronic pain states as well as desensitization of MORs (Campos-Jurado et al., 2019; Ozaki et al., 2002). Together, these data suggest that in chronic pain states, the potency or efficacy of MOR agonist induced effects could be decreased.

Therefore, the goal of this study was to evaluate the potency and effectiveness of MOR agonist-induced antihyperalgesic-like effects and rate decreasing effects before and after the induction of chronic neuropathic pain as well as over the course of 6 months of chronic neuropathic pain. Co-examination of suppression of rates of responding and analgesic effect is useful, as highly sedating drugs can produce a false positive in pain elicited tasks such as mechanical hypersensitivity (Stevenson et al., 2006; Negus et al., 2006). For these studies, we used the spared nerve injury (SNI) model (or sham control) because it produces a persistent hyperalgesic-like state that persists for at least 8 months (Decosterd & Wolfe, 2000; Erichsen & Blackburn-Munro, 2008). In addition, we evaluated these effects in in both male and female rats since few studies have evaluated the effects of MOR agonists in the SNI model and because women are more likely to experience neuropathic pain than men (Bouhassira, et al., 2008).

2.3 Methods.

Animals.

All rats were at least 8 weeks old at the start of the experiment. Rats were purchased from Envigo (Indianapolis, IN), and after delivery, were given one week to habituate to the animal housing facility. They were housed in standard cages with corncob bedding with water available ad libitum. There were 6 rats per condition in all experiments except one female rat was removed from the operant assay around the 4-6 month timepoint due to a prolapsed uterus. Rats were given enviropacks and wooden gnawing blocks for enrichment. The animal housing facility operates on a 12 hr light dark cycle, and all experiments were conducted during the light cycle. Rats in the mechanical hypersensitivity experiments were group-housed for the duration of the study with food and water available ad libitum. For the operant experiments, rats were single-housed and food

restricted with food restriction starting 24 hr prior to the start of operant training. Female rats were maintained on ~13 grams of standard chow, and male rats were maintained on ~18 grams of standard chow for the duration of the study, though water was available ad libitum.

SNI or sham surgery

Spared nerve injury surgery was performed as described by Decosterd & Woolfe (2000). Briefly, rats were deeply anesthetized with ketamine (90 mg/kg, intraperitoneal (i.p.)) and xylazine (10 mg/kg, i.p.). 5 mg/kg carprofen (subcutaneously (s.c.)) was given pre-emptively as well as 24- and 48-hr after surgery for post-operative pain relief. An incision was made in the left femoral muscle, exposing the sciatic nerve. A 2 mm section was removed from the peroneal and tibial nerves, while the sural was not manipulated. The remaining portions of the tibial and peroneal nerves were sutured to the muscle to minimize any incidence of nerve regrowth. The sham surgery consisted of only the incision into the femoral muscle without nerve manipulation. In both surgeries, the muscle and skin were closed separately with absorbable suture (5-0) and surgical staples, respectively.

Randall Selitto

Prior to any evaluation of mechanical threshold, all rats were habituated to the hammock-like restraint over three days for 5, 10, and 15 min respectively. For mechanical threshold evaluations, the paw pressure applicator (Randall Selitto, IITC Life Sciences, Woodland Hills, CA) was applied continuously with increasing pressure to the contralateral and ipsilateral hind paws until withdrawal or retraction occurred. The Analgesiometer recorded the pressure at which

hind paw withdrawal or retraction from the applicator occurred. The maximum cutoff used was 300 g of pressure.

Hot plate

Rats were placed on a 52°C hotplate (Columbus Instruments, Columbus, OH) and the latency to lick a hind paw was recorded. The maximum allowed time on the hotplate was 45 seconds, and if the rat did not lick a hind paw or jump off the hotplate by 45 seconds, the rat was removed and the latency of 45 seconds was recorded.

Schedule Maintained Responding

Equipment

All experiments were conducted in 18 standard MedPC operant chambers. These chambers were equipped with two nosepokes (ENV-114BM), a pellet dispenser (ENV-203-45), and a food hopper (ENV-200R2M). The food hopper was located in the middle of the right side of the chamber with nose pokes on either side. The active nosepoke was illuminated and responding on the active nosepoke resulted in sucrose pellet delivery and illumination of the house light. The inactive nosepoke was not illuminated, and responding on the inactive nosepoke was recorded but had no scheduled consequences.

Operant Training

Animals were trained to respond for 45 mg unflavored sucrose pellets (Bioserv, Flemmington, NJ) on a fixed ratio (FR)1 schedule of reinforcement in 20-min training sessions. Following each reinforcer delivery, there was a 10 sec timeout. The work requirement was

gradually increased to a FR5 schedule of reinforcement. Then, the pre-session blackout was gradually increased from 0-15 min, and response periods decreased from 20 to 3 min. The final procedure consisted of three, consecutive components in which each component consisted of a 15-min blackout and a 3-min response period. Responding on the active nosepoke only resulted in sucrose pellet delivery during the 3-min response period.

Testing

Testing was initiated after responding in all 3 components was stable across 5 consecutive days, defined as <30% in response rate between any two consecutive days. After testing was initiated at least two consecutive days of stable responding between tests were necessary. However, if responding deviated more than 30% between days, then three subsequent, consecutive days of stable responding were needed to proceed to the next test day. On average, 1-2 tests occurred per week.

Drugs

Fentanyl, nalbuphine, cocaine and THC were purchased from Sigma Aldrich (Burlington, MA). Morphine was purchased from Henry Schien (Novi, MI). SNC80 was generously gifted by Kenner C. Rice. NTX, diclofenac, quinpirole, and EKC were purchased from Tocris (Bristol, UK). Ketamine hydrochloride and xylazine were obtained from Hospira, INC (Lake Forest, IL). Carprofen (Rimadyl) was obtained from (Zoetis, Parsippany, NJ).

Fentanyl citrate, morphine sulphate, nalbuphine hydrochloride, naltrexone hydrochloride, cocaine hydrochloride, quinpirole hydrochloride, and ethylketazocine (EKC) were dissolved in sterile saline. SNC80 was dissolved in 3-5% 1 M HCl and the remainder of the solution was sterile

water. Δ-9-tetrahydrocannabidiol (THC) was dissolved in a 10:10:80 mixture of ethanol, DMSO, and sterile water. Diclofenac sodium was dissolved in sterile water. Morphine was purchased as a 50 mg/mL solution and diluted in sterile saline to relevant concentrations. THC, diclofenac, cocaine, EKC, nalbuphine, and quinpirole were given intraperitonially (i.p.), and all other drugs were given subcutaneously (s.c.).

In operant, Randall Selitto, and hot plate experiments, all drugs were administered cumulatively. In Randall Selitto and hot plate experiments, drugs were administered 5-7 days apart to minimize tolerance from repeated administration. In operant assays, opioids were not tested more than once in the same week.

Statistical Analyses

All ANOVA analyses were conducted in SPSS version 29. Datasets with multiple timepoints were analyzed with four-way repeated measures ANOVAs with sex, time, dose, and surgical status (SNI, sham) as independent variables and time and dose as repeated measures. Datasets with only one timepoint were analyzed with three-way repeated measures ANOVAs with sex, dose, and surgical status as independent variables. For all ANOVAs, corrected models were used in line with Mauchly's W criteria. Partial eta squared was calculated as an estimate of effect size. For main effects of dose, post-hoc one-way ANOVAs were used to determine which doses were statistically different from vehicle, and these analyses were split by other significant main effects (e.g., sex, time, or surgical status).

2.4 Results

Figure 2-1. Evaluation of paw withdrawal thresholds and body weight over 6 months of sham or SNI states.

In the absence of surgical manipulation, baseline withdrawal thresholds did not change over 6 months [no main effect of time: $F(5, 50)=1.96$, $p=0.10$, $n2p=0.16$] and were not different between males and females [no main effect of sex: [$F(1, 10)=3.9$, $p=0.08$, $n2p=0.28$]) (Figure 2-1A). Importantly, withdrawal thresholds in the naïve rats (Fig. 2-1A) were similar to those observed in the sham-treated rats (Fig. 2-1B).

As shown in Figure 2-1B, paw withdrawal thresholds were lower in the SNI groups than the sham groups [main effect, surgical status: $F(4.02, 120)=8.09$, $p<0.001$, $n2p=0.29$] and altered over time [main effect, time: $F(4.02, 120)=11.52$, $p<0.001$, $n2p=0.37$]. Specifically, there was a decrease in paw withdrawal thresholds following SNI, but not sham surgery, as evidenced by a time by surgical status interaction [$F(4.02, 120)=8.09$, $p<0.001$, $n2p=0.29$] Lastly, in both sham and SNI groups, there was no sex difference in paw withdrawal thresholds over time reflected by a no main effect of sex [$F(1, 20)=0.006$, $p=0.94$, $n2p=0$] and a no interaction of time and sex [$F(4.02, 120)=1.48$, $p=0.22$, $n2p=0.07$] (Fig. 2-1B).

To further understand the changes in paw withdrawal over time in both sham and SNI groups (Fig. 2-1B), a one-way ANOVA was run, split by surgical status. The one-way ANOVA was not significant in the sham group [$F(6, 77)=1.10$, $p=0.37$, $n2=0.08$]. Post-hoc analyses indicated that no post-surgical timepoint was significantly different than the pre-surgical value (all p's >0.86). The one-way ANOVA in the SNI group was significant, suggesting that the paw withdrawal thresholds changed over time [$F(6, 77)=17.17$, $p<0.001$, $n2=0.57$]. All timepoints were significantly different than pre-surgical baselines (p's<0.001).

Prior to surgery, fentanyl, morphine, and nalbuphine dose-dependently increased paw withdrawal thresholds prior to surgery (Fig. 2-1C). Prior to surgery, fentanyl dose dependently increased paw withdrawal thresholds, reflected by a main effect of dose [$F(2.82, 56.40)=336.33$, $p<0.001$, $n2p=0.944$]. There was no main effect of sex [$F(1, 20)=0.03$, $p=0.80$, $n2p=0.003$] or surgical status [$F(1, 20)=0.11$, $p=0.75$, $n2p=0.002$] in fentanyl-induced antinociceptive-like effects. All interaction terms failed to reach significance (p's>0.12). Morphine dose dependently increased paw withdrawal thresholds prior to surgery, reflected by a main effect of dose $F(1.75, 35.04)=124.86$, $p<0.001$, $n2p=0.86$] (Fig. 2-1C). There was no main effect of sex [$F(1, 20)=0.007$, $p=0.93$, $n2p=0$] or surgical status [$F(1, 20)=0.006$, $p=0.94$, $n2p=0$]. All interaction terms failed to reach significance (p's>0.64). Nalbuphine also dose-dependently increased paw withdrawal thresholds prior to surgery, reflected by a main effect of dose [$F(5, 100)=80.28$, $p<0.001$, $n2p=0.8$]. There was no main effect of sex [$F(1, 20)=0.06$, $p=0.47$, $n2p=0.03$] or surgical status [$F(1, 20)=0.35$, $p=0.56$, $n2p=0.02$]. All interactions failed to reach significance (p's>0.13).

As shown in Figure 2-1D, SNI-induced hypersensitivity did not alter weight gain in male or female rats as compared to the sham groups. There was a main effect of sex, such that females weighed less than males, as expected [$F(1, 19)=146.48$, $p<0.001$, $p=0.89$]. There was a significant main effect of time [$F(2.5, 47.48)=69.69$, $p<0.001$, $n2p=0.79$], such that rats gained weight over time. There was also a significant interaction of time and sex [$F(2.5, 47.48)=6.85$, $p<0.001$, $n2p=0.27$], reflecting differences in weight gain by sex. Interestingly, there was no main effect of surgery status [$F(1, 19)=0.04$, $p=0.85$, $n2p=0.002$] and no significant interaction between time and surgical status [$F(2.5, 47.48)=6.85$, $p<0.001$, $n2p=0.27$], suggesting that SNI and sham groups gained weight over time to the same extent. All other interaction terms failed to reach significance (p's>0.10).

Figure 2-2. MOR agonist-induced antinociceptive- and antihyperalgesic-like effects over 6 months of sham or SNI states.

Fentanyl dose dependently increased paw withdrawal thresholds, supported by a main effect of dose [$F(2.4, 46.21)=477.88$, $p<0.001$, $n2p=0.96$] (Fig. 2-2A, D). There was a main effect of surgical status [$F(1, 19)=205.02$, $p<0.001$, $n2p=0.92$]. Further, there was a significant dose by surgical status interaction [$F(2.4, 46.21)=24.48$, $p<0.001$, $n2p=0.56$]. These data indicate that paw withdrawal thresholds were lower in SNI as compared with sham rats, as expected, and suggest that fentanyl produced a larger increase in withdrawal thresholds in SNI rats as compared with that observed in sham groups (Fig. 2-2A, D). To determine which doses were significantly more effective than vehicle, one-way ANOVAs with Tukey's post hoc analyses, split by surgical status, revealed that doses of 0.032-0.1 mg/kg significant increased paw withdrawal thresholds in sham (all $p<0.001$) and SNI (all $p<0.001$) rats. There were no main effects of sex [$F(1, 19)=1.48$, $p=0.24$, $n2p=0.07$] or time [$F(1.4, 26.82)=1.73$, $p=0.20$, $n2p=0.08$]; however, there was a significant time by fentanyl dose interaction [$F(8, 152)= 4.36$, $p<0.001$, $n2p=0.18$] and time, dose, and sex interaction [$F(8, 152)=1.86$, $p=0.03$, $n2p=0.11$]. These interactions likely reflect minor, but statistically significant, decreases in the effects of 0.056 mg/kg fentanyl over time in the male sham and SNI groups and 0.032 and 0.056 mg/kg fentanyl in the female sham group. All other interactions failed to reach significance ($p's> 0.07$).

In Figure 2-2B and 2-2E, morphine produced dose dependent increases in paw withdrawal thresholds, supported by a main effect of dose [$F(3, 57)=430.31$, $p<0.001$, $n2p=0.96$], with 3.2 and 10 mg/kg morphine increasing withdrawal thresholds across all treatment groups (see Table 1). There was also a significant main effect of surgical status [$F(1,19)=186.74$, $p<0.001$, $n2p=0.91$] and a significant interaction between surgical status and dose [$F(3, 19)=25.75$, $p<0.001$,

n2p=0.58]. These data indicate that paw withdrawal thresholds are lower in SNI groups as compared with sham rats, as expected, and suggest that morphine produced a larger change in withdrawal thresholds in SNI rats as compared with that observed in sham groups. Morphine was slightly more potent in females, which is supported by a main effect of sex [F(1, 19)=11.99, p=0.003, n2p=0.39]. The potency and effectiveness of morphine did not change over time, supported by no significant main effect of time [F(1, 19)=0.016, p=0.90, n2p=0.001] or dose by time interaction [F(3, 57)=1.54, p=0.22, n2p=0.08]. All other interactions failed to reach significance (p's>0.18).

As shown in figures 2-2C and 2-2F, nalbuphine dose-dependently increased paw withdrawal thresholds, which was supported by a main effect of dose [F(2.5, 45.02)=62.19, p<0.001, n2p=0.78]. Unlike fentanyl and morphine, nalbuphine did not produce a maximal increase (e.g., at cutoff) in withdrawal threshold, consistent with a partial agonist. Interestingly, there was a main effect of surgical status [F(1, 18)=458115.32, p<0.001, n2p=0.70] but no interaction between surgical status and dose. These analyses suggest that, as expected, paw withdrawal thresholds were lower in SNI as compared with sham rats; however, nalbuphine produced similar effects in both sham and SNI rats in contrast to that observed with fentanyl and morphine.

There was a main effect of time [F(1, 18)=25.30, p<0.001, n2p=0.58], suggesting that nalbuphine was more effective at 6 months than 3 months (Fig. 2-2D, H). This is supported by a time by dose interaction [F(2.92, 52.58)=8.14, p<0.001, n2p=0.31]. There was no main effect of sex [F(1, 18)=0.93, p=0.35, n2p=0.05], suggesting that there were no sex differences in the effects of nalbuphine. However, there was a dose by sex by surgical status three way interaction [F(2.5,

45.02)=3.49, p=0.03, n2p=0.16] that likely reflects the increased effect of 32 mg/kg nalbuphine in female sham group compared with the male sham group.

To determine which doses of nalbuphine were altered by time, a one-way ANOVA split by surgical status and time was run. The results are displayed in table 2; bold values are significant. 1-3 months following surgical manipulation, there was a main effect of dose and post hoc analyses determined that 10 and 32 mg/kg nalbuphine were significantly more effective than vehicle in all groups (Table 2-2). However, 4-6 months following surgical manipulation, 3.2-32 mg/kg nalbuphine were significantly more effective than vehicle in all groups (Table 2-2) suggesting that nalbuphine was somewhat more potent over time in SNI and sham groups.

Figure 2-3. Acute antinociceptive effects of opioid analgesics in the hot plate assay

In hotplate experiments, fentanyl dose dependently increased the latency to respond in both male and female rats (Fig. 2-3A, F) [main effect, dose: F(3.02, 80)=148.72, p<0.001, n2p=0.88]. There was no main effect of surgical status [F(1, 20)=0.021, p=0.89, n2p=0.001] or interaction between dose and surgical status [F(3.02, 80)=0.51, p=0.68, n2p=0.14] suggesting the potency of fentanyl was not altered by surgical status. There was a significant main effect of sex [F(3.02, 80)=8.88, p=0.007, n2p=0.31], and interaction between sex and dose [F(1, 20)=0.021, p=0.89 n2p=0.001], indicating that fentanyl was more potent in male rats, specifically 0.032 mg/kg fentanyl. Within each sex, a one way ANOVA for dose found that the main effect of dose is significant in males [F(4, 55)=43.54, p<0.001, n2=0.76] and females [F(4,55)=88.03, p<0.001, n2=0.87]. In males, 0.032 (p=0.008), 0.056 (p=0.002), and 0.1 mg/kg (p<0.001) were significantly more effective than vehicle. In females, only 0.1 mg/kg was significantly more effective than

vehicle ($p<0.001$). Therefore, SNI-induced hyperalgesia did not alter baselines or fentanyl-induced antinociceptive-like effects.

Morphine dose dependently increased the latency to respond in hot plate assays in both sexes (Fig. 2-3C, H) [main effect, dose: $F(1.80, 36.02)=203.99$, $p<0.001$, $n2p=0.91$]. The main effect of dose is significant in both males [$F(3, 44)=186.84$, $p<0.001$, $n2=0.93$] and females [$F(3, 44)=49.84$, $p<0.001$, $n2=0.77$]. In males, 3.2 and 10 mg/kg morphine were more effective than vehicle ($p<0.001$). In females, 1 ($p=0.003$), 3.2 ($p<0.001$), and 10 ($p<0.001$) mg/kg morphine were more effective than vehicle. There was no main effect of surgical status [$F(1, 20)=2.98$, $p=0.10$, $n2p=0.13$] and no surgical status by dose interaction [$F(3, 80)=1.15$, $p=0.33$, $n2p=0.04$], further suggesting that SNI did not alter the potency of morphine. There was a significant sex difference, indicating that morphine was more potent in females than males [main effect, sex: $F(1, 20)=45.56$, $p<0.001$, $n2p=0.70$]. These data were further supported by a significant interaction of sex and dose, such that 3.2 and 10 mg/kg morphine were differentially effective in males and females [$F(1.80, 36.02)=7.13$, $p<0.003$, $n2p=0.26$]. All other interactions failed to reach significance ($p's> 0.17$).

On the hotplate, morphine-induced antinociceptive effects were blocked by a pretreatment of 0.3 mg/kg NTX (Fig. 3D, I). There was a significant main effect of pretreatment such that NTX decreased the latency to respond in both males and females [$F(1, 40)=345.13$, $p<0.001$, $n2p=0.90$]. There was also a significant main effects of sex and a significant NTX pretreatment by sex interaction, such that NTX pretreatment blocked morphine-induced antinociceptive effects to a greater extent in males (average = 10.84) than females (average = 23.34) [$F(1, 40)=17.33$, $p<0.001$, $n2p=0.30$].

On the hotplate, nalbuphine did not alter response latency in either males or females up to doses of 32 mg/kg [main effect, dose [$F(3, 60)=0.90$, $p=0.45$, $n2p=0.04$], regardless of surgical

status [no main effect, surgical status: F(1, 20)=2.09, p=0.16, n2p=0.09] (Fig. 3E, H). All other main effects failed to reach significance (p's> 0.20). Further, all interaction terms failed to reach significance (p's >0.26).

Figure 2-4. Effects of other (non-MOR agonist) analgesics on paw withdrawal thresholds.

In Randall Selitto experiments, SNC80 (Fig. 2-2A, F) failed to alter paw withdrawal thresholds, reflected by a main effect of dose [F(3, 60)=1.86, p=0.15, n2p=0.0]. However, there was a main effect of surgical status [F(1, 20)= 14.96, p<0.001, n2p=0.43], suggesting that the SNI groups had lower paw withdrawal thresholds than sham groups.

EKC (Fig. 2-4B, G) dose dependently increased paw withdrawal pressures in Randall Selitto assays, reflected in a significant main effect of dose [F(2.98, 59.45)=86.57, p<0.001, n2p=0.81]. While there was no main effect of sex, there was a significant three-way interaction between dose, surgery status, and sex [F(2.97, 59.45)=3.77, p=0.02, n20=0.16]. This demonstrates that EKC was approximately equally effective in the sham groups, regardless of sex, while EKC was more effective in the male SNI group than the female SNI group. Doses of 0.01 and 0.032 mg/kg EKC increased withdrawal threshold in both male and female SNI rats, though EKC was slightly more potent in sham groups with 0.0032-0.032 mg/kg producing antinociceptive-like effects in both males and females (2-4E, J).

In Randall Selitto experiments, diclofenac dose dependently increased paw withdrawal thresholds in SNI groups, reflected by a significant main effect of dose [F(4, 100)=7.81, p<0.001, n2p=0.24], and there was no sex difference [main effect: sex, F(1, 20)= 0.01, p=0.92, n2p=0.001] (Fig. 2-4C, H). There was a significant main effect of surgical status [F(1, 20)= 136.87, p<0.001, n2p=0.87]. To determine which doses of diclofenac altered paw withdrawal thresholds, a one-way

ANOVA was run, split by surgical status. In the sham groups, there was no main effect of dose [$F(4, 55)=1.44$, $p=0.23$, $n2=0.095$]. In the SNI groups, there was a main effect of dose [$F(4, 55)=7.80$, $p<0.001$, $n2=0.37$]. Tukey's post hoc analysis revealed that 10 ($p<0.001$) and 32 ($p<0.001$) mg/kg diclofenac significantly increased paw withdrawal thresholds in SNI groups compared to vehicle.

Gabapentin dose dependently increased paw withdrawal thresholds in Randall Selitto assays [main effect of dose $F(2.78, 55.65)=64.66$, $p<0.001$, $n2p=0.77$], and there was no sex difference [main effect of sex $F(1, 20)=0.85$, $p=0.37$, $n2p=0.04$] (Fig. 2-4E, J). There was no change in the effects of gabapentin over time, reflected by no main effect of time [$F(1, 20)=2.09$, $p=0.16$, $n2p=0.10$], no time by sex interaction [$F(1, 20)=0.05$, $p=0.82$, $n2p=0.003$], and no time by surgical status interaction [$F(1, 20)=2.15$, $p=0.16$, $n2p=0.24$]; however, there was a time, sex, and surgical status interaction such that gabapentin was slightly less potent in males at 3 months vs 6 months, while there were no differences in effectiveness in the female SNI group [$F(1, 20)=6.36$, $p=0.02$, $n2p=0.24$]. To determine which doses of gabapentin were significantly more effective than vehicle, a one-way ANOVA was used to determine the main effect of dose in both sham and SNI groups. The one-way ANOVA was significant in sham groups [$F(4,55)=2.63$, $p=0.04$, $n2=0.16$]; however, Tukey's post hoc analyses revealed that all doses tested failed to produce significantly different effects than vehicle. 180 mg/kg was close to the threshold for significance ($p=0.06$), however, the difference between the effects of 180 mg/kg and vehicle are not statistically different. In the SNI group, the one-way ANOVA found a significant main effect of dose [$F(4,55)=5.03$, $p=0.002$, $n2=0.27$], and Tukey's post hoc analyses determined that 180 mg/kg gabapentin significantly increased paw withdrawal threshold compared to vehicle in SNI rats.

Figure 2-5. Effects of cocaine and quinpirole on paw withdrawal thresholds

In Randall Selitto experiments, quinpirole produced dose-dependent increases in the paw withdrawal thresholds, supported by a main effect of dose [$F(3.00, 59.88)=38.23$, $p<0.001$, $n2p=0.66$], with significant increases in withdrawal threshold as compared with vehicle at 0.1 mg/kg ($p=0.002$, sham rats) or 0.32 and 1 mg/kg (both $p<0.001$, SNI rats) (Fig. 2-5A, C). There was a main effect of surgical status as expected [$F(1, 20)=47.33$, $p<0.001$, $n2p=0.7$], but there was no main effect of sex [$F(1, 20)=0.06$, $p=0.80$, $n2p=0.003$]. All interaction terms failed to reach significance, including the three-way interaction ($p>0.36$).

In Randall Selitto experiments, cocaine dose dependently increased paw withdrawal thresholds [main effect of dose [$F(2.44, 48.84)$)$=18.57$, $p<0.001$, $n2p=0.48$] (Fig. 2-5B, D). As expected, there was a significant main effect of surgical status [($F(1, 20)=72.90$, $p<0.001$, $n2p=0.79$]. There was a significant sex difference [main effect of sex [$F(1, 20)=6.98$, $p=0.02$, $n2p=0.26$] such that cocaine was more effective in female rats, regardless of surgical condition. Further, there was a three way interaction between dose, surgical status, and sex [$F(2.44, 48.84)=3.23$, $p=0.04$, $n2p=0.14$] such that cocaine was more effective in the female SNI group than any other group (Fig. 2D). To further analyze the effects of cocaine, one-way ANOVAs were used to compare the effects of different cocaine doses within each sex and surgical status group. There was no main effect of dose in the female sham group [$F(3, 20)=0.53$, $p=0.67$, $n2=0.07$] or male sham group [$F(3, 20)=1.20$, $p=0.33$, $n2=0.15$], suggesting that cocaine did not alter paw withdrawal thresholds in sham rats regardless of sex. There was a significant main effect of dose in the female SNI group [$F(3, 20)=45.47$, $p<0.001$, $n2=0.87$], with significant increases in withdrawal threshold at 18 and 32 mg/kg (both $p<0.001$). There was a significant main effect of

dose in the male SNI group [F(3, 20)=8.39, p<0.001, n2=0.56], with significant increases in withdrawal threshold at 32 mg/kg (p=0.002).

Figure 2-6. Drug-free rates of responding prior to and after sham or SNI surgery

Rates of responding were not altered by sham or SNI surgery in males (Fig. 2-6A) or females (Fig. 2-6B). As shown in figure 6, sham or SNI surgery did not alter rates of responding for sucrose pellets in male or female rats. There was no main effect of sex [F(1, 20)=0.07, p=0.79, n2p=0.004], surgery status, [F(1, 20)=2.58, p=0.12, n2p=0.11], or time (pre, post) (Fig. 2-6A, B). All interaction terms failed to reach significance (p's>0.14).

Figure 2-7. MOR agonist-induced rate suppressant effects

The effects of fentanyl on rates of responding are shown in Figure 2-7. Fentanyl dose dependently decreased rates of responding in both males (Fig. 2-7A, B) and females (Fig. 2-7C, D), reflected by a significant main effect of dose [F(2.52, 37.72)=154.35, p<0.001, n2p=0.91]. There was no main effect of time [F(2, 30)=0.87, p=0.43, n2p=0.06], surgical status [F(1, 15)=0.002, p=0.97, n2p=0], or sex [F(1, 15)=3.06, p=0.10, n2p=0.17]. All interaction terms failed to reach significance (p's >0.21). Analysis of data collapsed across time and sex show that fentanyl doses of 0.032-0.1 mg/kg significantly decreased rates of responding as compared with vehicle (Tukey's posthoc, p<0.001). Together, these data indicate that fentanyl-induced rate decreasing effects were not altered by SNI surgery for up to six months post-injury and there were no sex differences in the rate decreasing effects of fentanyl and up to 6 months of SNI induced hyperalgesic-like states.

The effects of morphine on rates of responding are shown in Figure 2-7. Morphine dose dependently decreased rates of responding in both males (Fig. 2-7A, B) and females (Fig. 2-7C, D). Analysis of data collapsed across time and sex show that morphine doses of 3.2 and 10 mg/kg significantly decreased rates of responding as compared with vehicle (Tukey's posthoc, $p<0.001$). There was no main effect of time [$F(2, 38)=2.92$, $p=0.07$, $n2p=0.13$], surgical status $F(1, 19)=0.0.003$, $p=0.96$, $n2p=0$], or sex [$F(1, 19)=0.28$, $p=0.60$, $n2p=0.02$]; however, there was a significant four-way interaction between time, sex, dose, and surgical status [$F(6, 114)=3.14$, $p=0.007$, $n2p=0.14$]. These analyses suggest that there were likely small changes in the effectiveness of single doses of morphine across the various conditions. For example, in males, 3.2 mg/kg morphine was less rate suppressant 1-3 or 4-6 months following SNI surgery and 4-6 months post-surgery in the sham group. In contrast, the effects of morphine are relatively unchanged in female rats over time. Overall, these results suggest that the rate decreasing effects of morphine were slightly less potent over time but perhaps only in male rats.

The effects of nalbuphine on rates of responding are shown in Figure 2-7. Since nalbuphine dose effect curves established 4-6 months post-surgery were substantially different from those measured pre-surgery and 1-3 months post-surgery, data analyses only directly compared nalbuphine dose effect curves established pre-surgery and 1-3 months post-surgery. Nalbuphine dose-dependently decreased rates of responding, as supported by a main effect of dose [$F(2.17, 26.09)=41.15$, $p<0.001$, $n2p=0.77$]. Further there was not a significant dose by surgical status interaction [$F(3, 26)=0.56$, $p=0.67$, $n2p=0.04$]. There was no main effect sex or surgical status, suggesting that there were no differences in the rate decreasing effects of nalbuphine between females and males and between sham and SNI conditions. There was a main effect of time [$F(2.34, 28.05)=3.82$, $n2p=0.24$] and a significant interaction of time and nalbuphine dose [$F(2.17,$

26.09)=41.15, p<0.001, n2p=0.77]. These data indicate that nalbuphine became less potent over time in both males and females independent of surgical status. Interestingly, 4-6 months after induction of sham (2-7A, C) or SNI (2-7B, D), nalbuphine was much less effective in decreasing rates of responding, with doses up to 100 mg/kg nalbuphine producing minimal, if any, rate decreasing effects. Overall, these data indicate that there were small changes in the rate decreasing effects of fentanyl and morphine and large rightward shifts in the effects of nalbuphine over time, and time, rather than nerve injury, produced significant changes in the rate decreasing effects of these MOR agonists. Overall, nalbuphine induced rate suppressant effects were subject to large changes over time, however, these changes are not explained by SNI-induced hypersensitivity.

Figure 2-8. Rate suppressant effects of cocaine and gabapentin

As shown in Figure 2-8, cocaine dose-dependently decreased rates of responding (Fig. 2-8A-D) prior to surgery, 1-3 months, and 4-6 months after both SNI or sham surgeries supported by a main effect of dose [F(2.05, 22.59)=63.05, p<0.001, 0.85], with significant rate decreasing effects at 18 and 32 mg/kg as compared with vehicle (p<0.001 both). All other main effects and interactions failed to reach significance (p's >0.16). This suggests that cocaine induced rate suppressant effects were not altered over time or by nerve injury.

As shown in Figure 2-8(A-D), gabapentin decreased rates of responding, reflected by a main effect of dose [F(3, 26.15)=12.12, p<0.001, n2p=0.50]. There were no main effects of time [F(2, 24)=0.41, p=0.67, n2p=0.03] or surgical status [F(1, 12)=4.49, p=0.06, n2p=0.27], suggesting that generally, gabapentin-induced rate decreasing effects are not altered by nerve injury or over time. However, there was a main effect of sex [F(1, 12)= 10.29, p=0.008, n2p=0.46], a sex by time interaction [F(2, 24)=4.82, p=0.02, n2p=0.29], and a sex by time by surgical status

three-way interaction [F(6, 72)=4.03, p=0.002, n2p=0.09], indicating that gabapentin did not decrease rates of responding in male rats up to a dose of 180 mg/kg and that the gabapentin decreased response rates to a lesser extent over time in female sham rats as compared with female SNI rats.

2.5 Discussion

Previous studies have demonstrated that pain states, including neuropathic pain states, resulted in increased levels of endogenous opioid peptide release (Bruehl et al., 2012, 2013, 2014), decreased MOR expression (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Pol et al., 2006; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998), and decreased MOR activation (Campos-Juardo et al., 2019; Ozaki et al., 2002). However, we know relatively little about if chronic pain states alter MOR agonist-induced antinociceptive- or antihyperalgesic-like effects over time. Therefore, the goal of this study was to evaluate MOR agonist-induced antinociceptive-like effects, antihyperalgesic-like effects, and rate suppressant effects in injured animals and sham animals over 6 months of SNI-induced hypersensitivity or sham states.

In the present study, SNI surgery induced a persistent decrease in paw withdrawal thresholds from mechanical stimuli as compared with the sham control condition, suggesting long-lasting mechanical hypersensitivity following SNI surgery (Fig. 2-1). Acutely, fentanyl, morphine, and nalbuphine induced dose-dependent increases in the antinociceptive- and antihyperalgesic-like effects in Randall Selitto assays (Fig. 2-1C, 2-3). Unlike fentanyl and morphine, nalbuphine failed to produce a maximum effect (300 g paw withdrawal threshold) (Fig. 2-2C, F), which is consistent with partial agonist activity. Consistent with other partial agonists, nalbuphine failed to

produce antinociceptive-like effects in the hot plate assay (Fig. 2-3). Additional non-MOR agonist drugs with analgesic-like effects were tested in Randall Selitto assays. SNC80, a DOR agonist, failed to alter paw withdrawal thresholds, but EKC, a KOR agonist, produced dose-dependent antinociceptive- and antihyperalgesic-like effects in both sexes (Fig. 2-4). THC, diclofenac, and gabapentin produced antihyperalgesic-like effects but not antinociceptive-like effects (Fig. 2-4). Quinpirole produced dose dependent antinociceptive- and antihyperalgesic-like effects, and cocaine produced antihyperalgesic-like effects (Fig. 2-5).

To determine if chronic neuropathic pain altered the potency and or/efficacy of MOR agonist- induced antinociceptive- or antihyperalgesic-like effects, fentanyl, morphine, and nalbuphine were repeatedly assessed over 6 months following sham and SNI surgery. Over time, small shifts in the effects of individual doses of fentanyl and morphine were observed; however, this was observed independent of surgical status, suggesting that SNI-induced hypersensitivity does not explain the small changes in MOR agonist-induced effects. MOR agonists can produce robust behavioral disruption, and one measure of this is decreased rates of responding. Fentanyl, and morphine dose-dependently decreased rates of responding prior to surgery in all groups (Fig. 2-7). 1-3 months or 4-6 months after surgery, there were changes in the effectiveness of individual doses of fentanyl and morphine independent of surgical status; however, there were no large shifts in dose effect curves. These data collectively suggest that the behavioral effects of high efficacy MOR agonists were not altered by the presence of chronic neuropathic pain states. Though previous studies have demonstrated a decrease in MOR expression or function in pain states, it is possible this decrease is not sufficient to alter the effects of higher efficacy MOR agonists.

Unlike fentanyl and morphine, nalbuphine-induced antinociceptive- and antihyperalgesic-like effects were more potent 6 months postoperatively than 3 months in both male and female rats

(Fig 2-2C, F). Prior to surgery, nalbuphine dose-dependently decreased rates of responding in all groups (Fig. 2-7). 1-3 months after sham or SNI surgery, nalbuphine induced rate suppressant effects were less potent in both sham and SNI groups, and though there was not a dose by surgical status interaction, this was to a slightly greater extent in both male and female SNI groups compared to sham groups (Fig. 2-7). 4-6 months after surgery, larger doses were needed to decrease rates of responding (Fig. 2-7), and nalbuphine significantly decreased rates of responding in only the male sham group (Fig. 2-7A). Collectively, independent of surgical status, nalbuphine was less rate suppressant 4-6 months after surgery. The leftward shifts in nalbuphine-induced analgesic-like effects and rightward shifts in the rate suppressant effects were observed in both sham and SNI groups, which suggests that SNI-induced hypersensitivity did not induce changes in the potency of nalbuphine. This suggests other factors may underlie the observed shifts.

First, rats began these experiments age-matched and at approximately 8 weeks old. However, these studies are longitudinal and lasted approximately 9-11 months (see methods). Previous studies have demonstrated that nalbuphine-induced antinociceptive-like effects were more potent and efficacious in older rats (Smith & Gray, 2001). Second, it is also possible activity at other opioid receptors may explain increased sensitivity to nalbuphine over time. Nalbuphine has ~9x higher affinity for MOR than kappa opioid receptors (KOR); however, nalbuphine was more efficacious in a GTPγS assay at KORs (~47%) than MORs (~18%) (Elmariah et al., 2022). KOR agonists have been demonstrated to effectively attenuate neuropathic pain-like states in rodents (Hall et al., 2016; Paton et al., 2022). Third, nalbuphine induced rate suppressant effects decreased over time, and a decrease in behavioral disruption may explain increased sensitivity in the mechanical hypersensitivity experiments. Finally, it is somewhat unlikely that changes in nalbuphine effects are due to changes in MOR expression, as previous studies suggest a decreased

expression of MORs in pain states (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Pol et al., 2006; Porecca et al., 1998; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998). This would be consistent with the decreased rate suppressant effects; however, this is not consistent with increased sensitivity in Randall Selitto assays.

Previous work has demonstrated that tolerance develops differentially to MOR agonist-induced behavioral effects, such that tolerance to analgesic and euphoric effects of MOR agonists occurs prior to tolerance to respiratory depressant or constipating actions (Angst et al., 2009; Grunkemeir 2007; Galligan et al., 2014; Mohamed et al., 2013; Paronis & Holtzman, 1994; Ross et al., 2008; Shippenberg et al., 1988). Other studies have demonstrated a different level of receptor reserve for the analgesic and primary reinforcing effects of MOR agonists (Zernig, Lewis, & Woods, 1997). The results in the present study may therefore suggest that nalbuphine-induced rate suppressant effects of MOR agonists have a lower receptor reserve than those of nalbuphine-induced analgesic effects.

Overall, the present study found few sex differences in MOR agonist-induced antinociceptive- and antihyperalgesic-like effects. This was somewhat unexpected as many previous studies have demonstrated sex differences (for example: Barrett, Smith, & Picker, 2002; Cook et al., 2000; Cook & Nickerson, 2005; Craft et al., 2001; Peckham & Traynor 2005, 2006; Terner et al., 2002; 2005; for review- Craft et al., 2003). However, relatively few studies have directly examined MOR agonist induced antinociceptive- and antihyperalgesic-like effects in a neuropathic model of pain and studied sex differences. Generally, more robust sex differences are observed with partial agonists and with higher intensity noxious stimuli (Cook et al., 2000).

Finally, as expected, there were group differences (sham, SNI) in paw withdrawal thresholds in Randall Selitto assays (Fig 2-1B, 2-2); however, consistent with previous work (Wen et al., 2007), SNI induced hypersensitivity did not induce a change in latency to nocifensive behavior compared to sham animals (Fig. 2-3) in the hot plate assay. This suggests that SNI-induced chronic nerve injury leads to mechanical hypersensitivity but not hypersensitivity to heat. It is possible that while SNI surgery produced a long-lasting mechanical hypersensitivity, the SNI animals may not be in a pain state, but rather experiencing decreases in the function of the injured limb or other sensations such as numbness in the injured hind paw/limb. This is supported by lack of evidence of pain depressed operant responding in SNI groups compared to rates of responding prior to surgery and in sham groups (Fig. 2-6) as well as similar weight gain over time in sham and SNI groups (Fig. 2-1). Though, we found that THC, diclofenac, and gabapentin produced small but significant increases in paw withdrawal thresholds in only the SNI groups (Fig. 2-4), which is consistent with the antihyperalgesic-like effects of these drugs. THC, diclofenac, and gabapentin did not produce significant antinociceptive-like effects in the sham groups, suggesting that these drugs did not simply disrupt behavior. Gabapentin failed to alter rates of responding in male rats, while low doses (30 mg/kg) significantly increased rates of responding and larger doses (180 mg/kg) significantly decreased rates of responding in female rats. Pre-clinical studies are extremely useful for evaluating the effects of drugs in various experimental states; however, the present findings highlight the utility of concurrently examining behaviorally disrupting effects (e.g., rate suppression) and pain-related behaviors when using pain-elicited assays such as Randall Selitto or hot plate.

In conclusion, the present study evaluated the antinociceptive-like effects, antihyperalgesic-effects, and rate suppressant effects of opioid analgesics varying in potency and

efficacy along with select non-opioid analgesics in the presence or absence of SNI-induced hypersensitivity. The overall finding was that SNI-induced chronic hypersensitivity failed to alter the antinociceptive-like effects, antihyperalgesic-like effects, and rate suppressant effects of MOR agonists. Over time, few changes were observed in the behavioral effects of higher efficacy MOR agonists, such as fentanyl or morphine. However, over time, there was an increase in sensitivity to nalbuphine induced antinociceptive-like and antihyperalgesic-like effects while a corresponding decrease in sensitivity to nalbuphine induced rate suppressant effects was observed. Future studies should directly evaluate the abuse potential of partial MOR agonists in the presence or absence of chronic pain states.

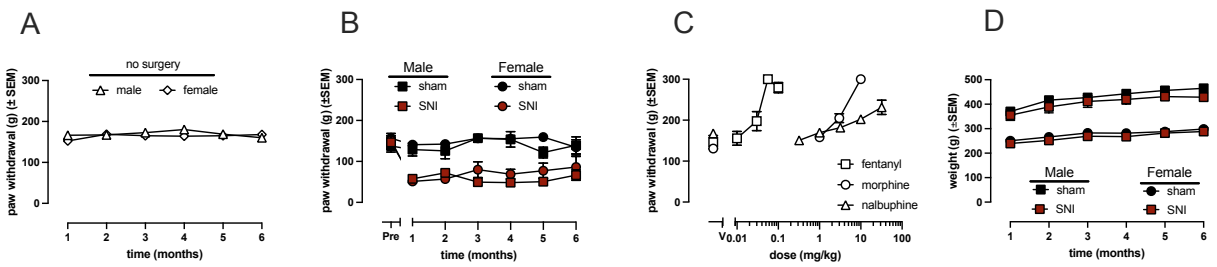

Figure 2-1. Paw withdrawal thresholds in the absence or presence of SNI-induced hypersensitivity, pre-surgical testing of opioid analgesics, and weights

Paw withdrawal thresholds were unchanged in naïve male and female rats over 6 months (A). In both males and females, following sham surgery, there was no significant change in paw withdrawal thresholds compared to pre-surgical values; however, following SNI surgery, there was a significant reduction in paw withdrawal thresholds that persisted for 6 months (B). Fentanyl, morphine, and nalbuphine produced dose dependent increases in paw withdrawal thresholds in all rats prior to surgery (should probably go before B), and data are plotted with male and females collapsed (C). There is no difference in the amount of weight gained between sham and SNI animals in either males or females over 6 months (D).

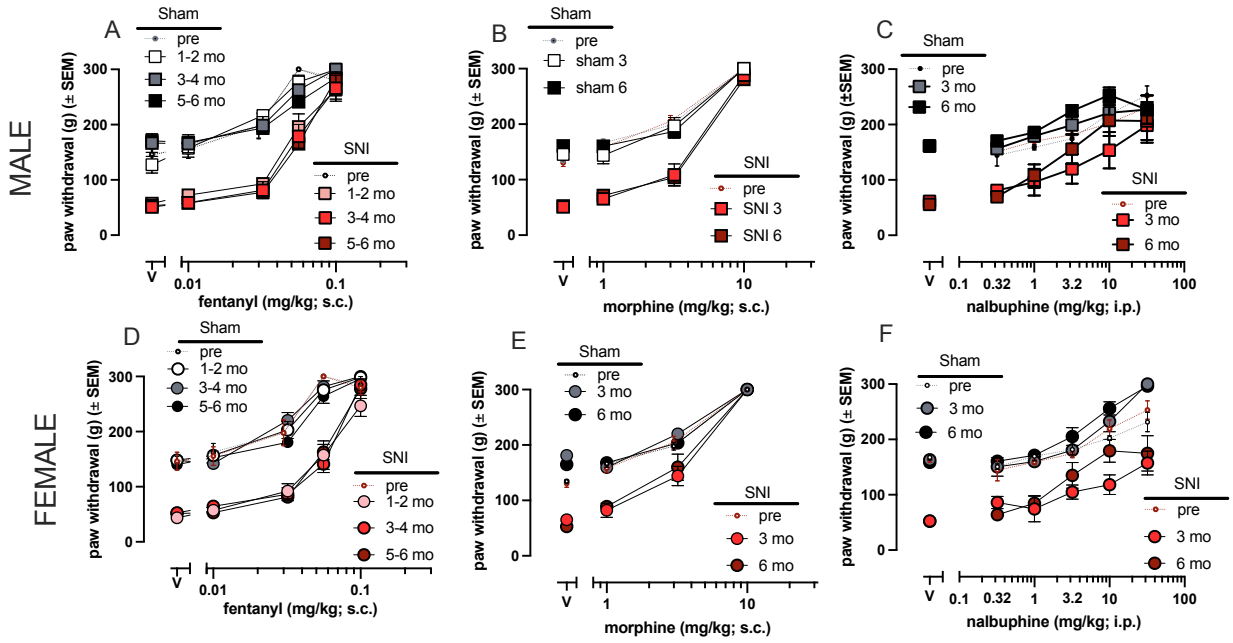

Figure 2-2. Effects of fentanyl, morphine, and nalbuphine on paw withdrawal thresholds

Fentanyl produced dose dependent increases in paw withdrawal thresholds in both males (A) and females (D) prior to surgery and following either sham or SNI; there were small, significant shifts in the dose effect curves in both sham and SNI groups, suggesting SNI did not alter fentanyl-induced effects. Morphine produced dose dependent increases in paw withdrawal thresholds in both males (B) and females (E) prior to surgery and following either sham or SNI; there were small, significant shifts in the dose effect curves in both sham and SNI groups, suggesting SNI did not alter morphine-induced effects.

Nalbuphine dose dependently increased in paw withdrawal thresholds in both males (C) and females (F) prior to surgery. Following either sham or SNI, there were leftward shifts in the nalbuphine dose effect curves over time, suggesting SNI did not alter nalbuphine induced effects.

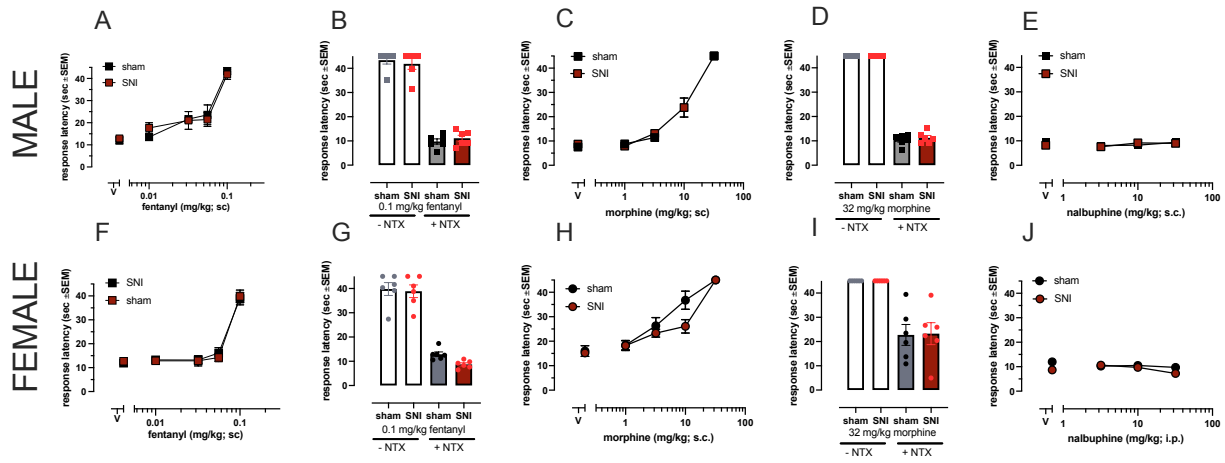

Figure 2-3. Effects of fentanyl, morphine, nalbuphine in the $52°C$ hot plate assay

Fentanyl produced dose-dependent antinociceptive-like effects in males (A) and females (F) regardless of surgical status. Pretreatment of naltrexone significantly reduced the antinociceptive effect of fentanyl in males (B) and females (G) regardless of surgical status. Morphine produced dose-dependent antinociceptive-like effects in males (C) and females (H) regardless of surgical status. Pretreatment of naltrexone significantly reduced the antinociceptive-like effect of morphine in males (D) and females (I), though naltrexone was significantly more effective in males. Nalbuphine failed to produce antinociceptive-like effects in either males (E) or females, regardless of surgical status (J). Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

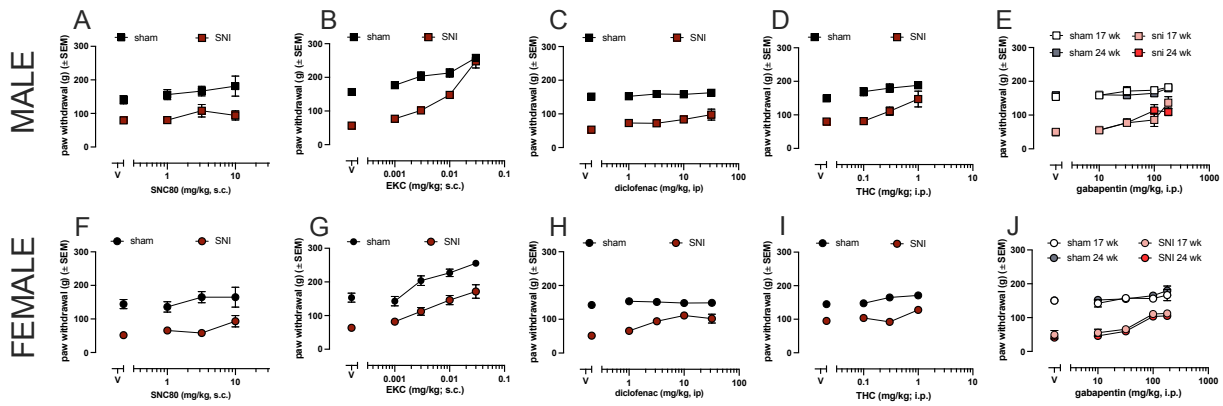

Figure 2-4. Effects of non-MOR agonist analgesics on paw withdrawal thresholds in the Randall Selitto assay

SNC80 failed to produce significant antihyperalgesic- or antinociceptive-like effects in both males (A) and females (F). EKC produced dose dependent antinociceptive- and antihyperalgesic-like effects in both males (B) and females (G). Diclofenac produced small, but significant antihyperalgesic-like effects in both males (C) and females (H) but did not produce significant antinociceptive-like effects in either sex (C, H). THC produced dose dependent antihyperalgesic-like and antinociceptive-like effects in both males (D) and females (I). Gabapentin produced dose dependent antihyperalgesic-like effects in both males (E) and females (J) but did not produce significant antinociceptive-like effects in either sex (E, J). There was no significant difference in the antinociceptive-like effects over time (E, J). Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

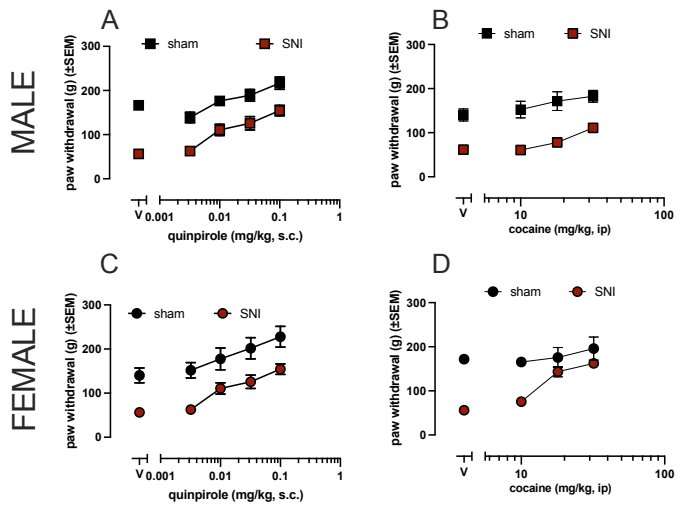

Figure 2-5. Effects of dopaminergic drugs on paw withdrawal thresholds

Quinpirole produced dose dependent antinociceptive-like and antihyperalgesic-like effects in both males (A) and females (C). Cocaine failed to produce antinociceptive-like effects in either males (B) or females (D). However, cocaine produced dose-dependent antihyperalgesic-like effects in both males (B) and females (D), though cocaine was significantly more effective in females. Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

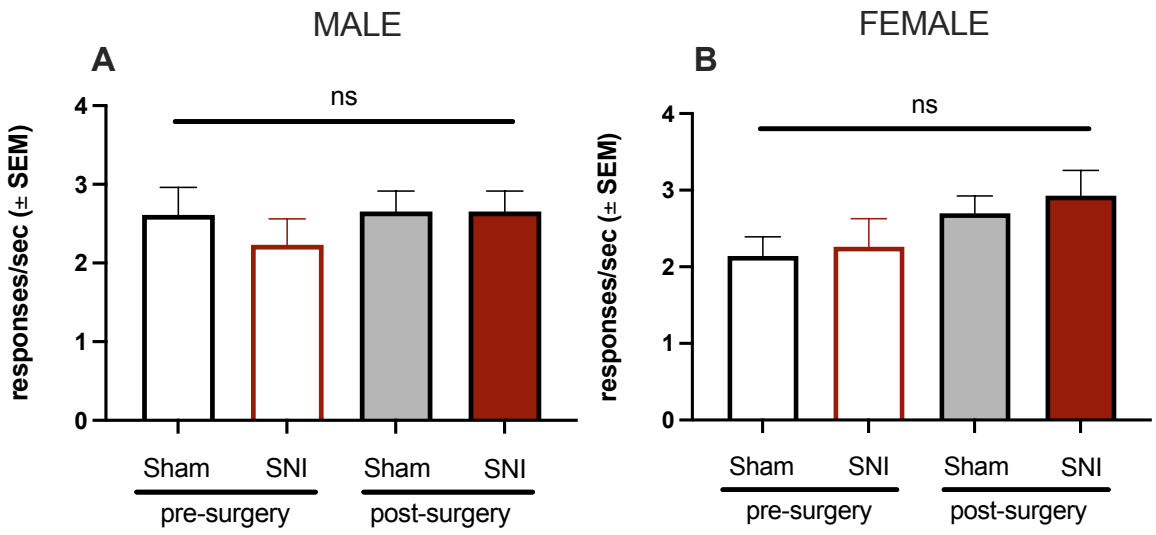

Figure 2-6. Drug free rates of responding prior to and after sham or SNI surgery

Prior to surgery, there were no differences in rates of responding between males (A) and females (B). Following sham or SNI surgery, there was no significant difference in rates of responding in either males (A) or females (B). Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

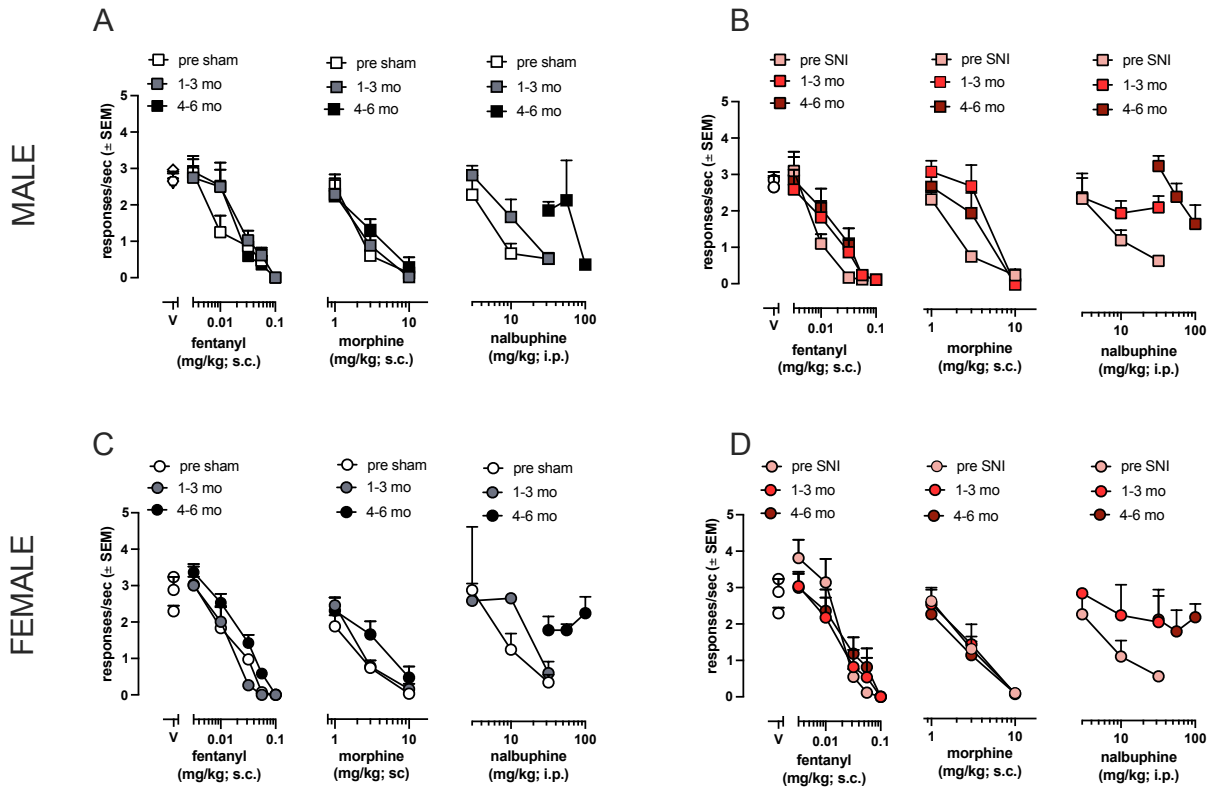

Figure 2-7. Rate suppressant effects of opioid analgesics in male and female rats prior to and 1-2 months or 3-4 months after sham or SNI surgery

Fentanyl dose-dependently decreased rates of responding prior to surgery in both males (A, B) and females (C, D). Following sham or SNI surgery, there were not significant shifts in the dose effect curves in males (A, B) or females (C, D). Morphine dose-dependently decreased rates of responding prior to surgery in both males (A, B) and females (C, D). After sham or SNI surgery, there were small, but significant shifts in the dose effect curves in both sham and SNI groups. Nalbuphine dose-dependently decreased rates of responding in males (A, B) and females (C, D). 1-3 months after surgery, nalbuphine was less potent in all groups, but still suppressed rates of responding. 4-6 months after surgery, up to 100 mg/kg nalbuphine failed to suppress rates of responding in any group except the male sham group (A). Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

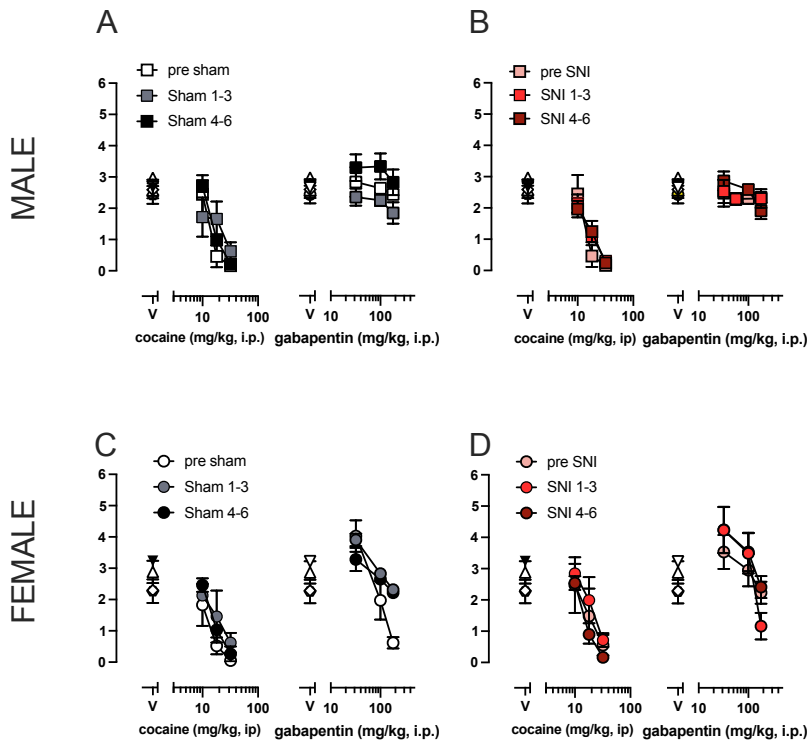

Figure 2-8. Rate suppressant effects of cocaine and gabapentin in male and female rats prior to and 1-2 months or 3-4 months following sham or SNI surgery

Prior to sham or SNI surgery, cocaine dose dependently decreased rates of responding in both males (A, B) and females (C, D). After sham or SNI surgery, there were no significant shifts in the dose effect curves in either males (A, B) or females (C, D). Gabapentin failed to alter rates of responding in male rats prior to either sham (A) or SNI surgery (B). There were no significant changes following sham (A) or SNI (B) surgery such that gabapentin did not alter rates of responding in male rats. In females, prior to and after either sham (C) or SNI (D) surgery, low doses of gabapentin (32 mg/kg) increased rates of responding compared to vehicle while larger doses (180 mg/kg) significantly decreased rates of responding compared to vehicle. There were no significant differences between sham (C) and SNI (D) groups or in dose effect curves over time following surgery. Data are plotted as the mean ± SEM. Data points are an average of 6 animals.

Table 2-1.	**Main effect: dose**		**Post-hoc analyses**		
Group	**$F_{(3, 20)}$**	**p**	**$p_{(0 \text{ vs } 1)}$**	**$p_{(0 \text{ vs } 3.2)}$**	**$p_{(0 \text{ vs } 10)}$**
Female sham	86.967	**<.001**	1	**0.008**	**<0.001**
Female SNI	121.998	**<.001**	0.62	**0.004**	**<0.001**
Male sham	231.163	**<.001**	0.56	**<0.001**	**<0.001**
Male SNI	101.426	**<.001**	0.32	**<0.001**	**<0.001**

Table 2-1. Post-hoc testing for main effect of dose of morphine in Figure 2B, D

This table displays the output from a one-way ANOVA analyzing the main effect of dose as well as Tukey's post-hoc testing for morphine induced antinociceptive- and antihyperalgesic-like effects in the Randall Selitto Assay (Fig. 2B, D). This one-way ANOVA was run with data collapsed across time following surgery, but data were split by sex and surgical status.

Table 2-2	Main effect: dose			Post-hoc analyses				
	Group	$F_{(5, 60)}$	p	$p_{(0 \text{ vs } 0.32)}$	$p_{(0 \text{ vs } 1)}$	$p_{(0 \text{ vs } 3.2)}$	$p_{(0 \text{ vs } 10)}$	$p_{(0 \text{ vs } 32)}$
1-3 mo	Sham	18.185	**<.001**	0.988	0.998	0.468	**<.001**	**<.001**
	SNI	9.14	**<.001**	0.78	0.737	0.084	**0.003**	**<.001**
4-6 mo	Sham	26.788	**<.001**	0.998	0.688	**<.001**	**<.001**	**<.001**
	SNI	17.363	**<.001**	0.989	0.326	**<.001**	**<.001**	**<.001**

Table 2-2. Post-hoc testing for main effect of dose of nalbuphine in Figure 2C, E

This table displays the output from a one-way ANOVA analyzing the main effect of dose as well as Tukey's post-hoc testing for nalbuphine induced antinociceptive- and antihyperalgesic-like effects in the Randall Selitto Assay (Fig. 2C, E). This one-way ANOVA was run with data collapsed across sex, but data were split by time following surgery and surgical status.

Table 2-3.	**Main effect: dose**		**Post-hoc analyses**		
Time	$F_{(5, 60)}$	p	$p_{(0 \text{ vs } 0.32)}$	$p_{(0 \text{ vs } 1)}$	$p_{(0 \text{ vs } 3.2)}$
Pre-surgery	43.154	<.001	0.496	<.001	<.001
1-3 months post	9.764	<.001	0.803	0.688	<.001

Table 2-3. Post-hoc testing for main effect of time in Figure 2-7 (nalbuphine)

This table displays the output from a one-way ANOVA analyzing the main effect of dose as well as Tukey's post-hoc testing for nalbuphine-induced rate suppressant effects in the schedule maintained responding assay (Fig. 7). This one-way ANOVA was run with data collapsed across sex and surgical status to evaluate the difference in nalbuphine-induced effect prior to and after surgery.

Chapter 3

The Effects of Chronic Neuropathic Pain-like States on the Reinforcing Effects of a Highly Potent MOR Agonist, Fentanyl

3.1 Abstract.

Chronic pain is a large public health problem in the U.S., as is opioid use disorder (OUD). There is a significant overlap in the chronic pain and OUD patient populations such that approximately 50-65% of chronic pain patients have OUD. Further, a recent study reported that opioid analgesics were used in nearly 70% of patients with chronic neuropathic pain. Some studies suggest chronic pain is a risk factor for development of OUD. Thus, the goal of this study was to determine if chronic neuropathic pain altered the ongoing self-administration of fentanyl, or a non-opioid drug of abuse, cocaine. Animals were trained to self-administer fentanyl or cocaine in a multi-dose self-administration procedure composed of five 25-min components, each exposing animals to a different dose of fentanyl (0-10 ug/kg/inf) or cocaine (0-0.56 mg/kg/inf). Once drug-maintained responding was stable, animals underwent either spared nerve injury (SNI) surgery to induce chronic neuropathic pain or sham states. Animals were allowed 72 hours of post-operative recovery and resumed self-administration studies on post-operative day 4. In fentanyl-trained animals, there was a significant decrease in fentanyl-maintained responding in both sham and SNI on post-operative day 4, compared to pre-surgical responding. Therefore, this decrease in fentanyl-maintained behavior is unlikely to be explained by the presence of SNI-induced hypersensitivity. By post-operative day 9, fentanyl-maintained responding had returned to pre-surgical levels. 2- to 4- weeks after surgery, there were not significant shifts in the dose effect curves in either sham or

SNI groups. However, there was an increased intake of specifically 10 ug/kg fentanyl, suggesting SNI-induced hypersensitivity is not responsible for this increase. Cocaine self-administration was not significantly altered by the presence of SNI or sham states at any timepoint tested. Collectively, these results suggest that chronic neuropathic pain failed to alter the reinforcing effects of fentanyl, or non-opioid drug of abuse, cocaine. Future studies should evaluate the abuse potential of lower efficacy MOR agonists such as nalbuphine or buprenorphine, as small changes were observed in fentanyl-maintained behavior over time in both SNI and sham groups.

3.2 Introduction

Both chronic pain and opioid use disorder (OUD) are substantial public health concerns with overlapping patient populations, such that 50-65% of chronic pain patients have been diagnosed with OUD (Hser et al., 2017; Latif et al., 2021). While mu opioid receptor (MOR) agonists are useful analgesics for moderate to severe pain, they are rarely considered first-line treatments for chronic pain because repeated use of MOR agonists leads to the development of tolerance and requires escalation of dose, physical dependence, and abuse liability This is particularly concerning since many (~69%) patients with chronic neuropathic pain are treated with MOR agonists at some point for pain management, and ~20% of chronic pain patients are treated with opioids persistently/frequently (Hoffman et al., 2017). While exposure to and use of MOR agonists increases abuse liability, some studies have also suggested that pain itself a risk factor for the development of OUD (Bauman et al., 2023; Brummet et al., 2017; Katz et al., 2013). However, we know relatively little about whether or not chronic pain alters the reinforcing effects of MOR agonists.

Previous preclinical studies have examined the rewarding or reinforcing effects of MOR agonists in the presence of various pain states, but the results have been heterogeneous. Some studies show an *increase* in the rewarding (Cahil et al., 2013; Lim et al., 2014; Navratilova et al., 2020; Zhang et al., 2014) or reinforcing effects (Colpaert et al., 2001; Hippolito et al., 2015; Higginbotham et al., 2023) of MOR agonists in the presence of a pain state, while other pre-clinical experiments have shown a *decrease* in rewarding or reinforcing effects of opioids (Ozaki et al., 2002; Suzuki et al., 1996; Nazarin et al., 2021; Narita et al., 2005; Wade et al., 2003; Martin et al., 2007). Further, other studies showed *no impact* of pain states on opioid induced reinforcing effects (Barattini et al., 2023; Hou et al., 2015; Reiner et al., 2021) or opioid rewarding effects (Shippenberg et al., 1988). In previous self-administration studies specifically, there are many methodological differences; however, one difference worth considering is the duration of the pain state used. Many previous studies have utilized acute or sub-chronic pain states (Baratinni et al., 2023; Hippolito et al., 2015; Higginbotham et al., 2023; Hou et al., 2015; Reiner et al., 2021; Wade et al., 2003), which prevents the long-term evaluation of MOR agonist-maintained behavior.

In addition, only three studies have assessed if pain states alter established, ongoing opioid self-administration (Baratinni et al, 2023; Neelakanten et al., 2017, Reiner et al., 2021). Two of these studies utilized short acting (lactic acid, capsaicin; Reiner et al., 2021) pain states or sub-chronic pain states (CFA; Baratinni et al., 2003), and both of these studies found no effect of pain state on MOR agonist-maintained behavior. The third used paclitaxel-induced neuropathic pain and investigated if developing neuropathic pain altered breakpoint responding for morphine infusions in mice (Neelakanten et al., 2017). This study found that the injured animals had consistent, stable breakpoint responding for morphine, while the saline-treated group displayed a decrease over time. However, no study has examined the impact of a fully developed, chronic

neuropathic pain state on the ongoing self-administration of opioids. Therefore, the goal of this study was to examine (within-subject) the reinforcing effects of fentanyl before and after the induction of a persistent, long-lasting pain state caused by the spared nerve injury (SNI).

3.3 Methods

Animals

All rats were at least 8 weeks old at the beginning of the study. Rats were ordered from Envigo labs (Indianapolis, IN). Following delivery, rats were given one week to habituate to the animal housing facility. All rats were housed in standard cages with corncob bedding. Water was available ad libitum. The housing facility was kept on a 12-hr light dark cycle, and all experiments were conducted in the light cycle.

Following intravenous catheter implant, rats were single housed for the remainder of the study of the study. These rats were food restricted 24 hours prior to the start of self-administration experiments. Males were fed 5 pellets and females were fed 4 pellets of standard rat chow (5LOD, LabDiet) to maintain rats at approximately 90% of their free-feeding body weight. Following SNI or sham surgery, rats were given wooden gnawing blocks and enviropacks as enrichment.

For the Randall Selitto assays, rats were single housed for 7-10 days following surgery (femoral catheter implant & SNI or sham surgery performed on the same day) until sutures were removed, then were group housed for the remainder of the study with food available ad libitum. Following surgery, rats were given wooden gnawing blocks and enviropacks as enrichment.

Surgery

Anesthesia

For all surgeries, rats were deeply anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine, injected intraperitonially (i.p.). Rats were given carprofen (5 mg/kg) subcutaneously (s.c.) prior to surgery as well as 24-hr later for pre- and post-operative analgesia. Following SNI or sham surgery, rats were given 5 mg/kg carprofen s.c. 24- and 48-hr after surgery.

Femoral Vein Catheter and Backplate Implant

Rats were implanted with a chronic, indwelling intravenous catheter. Briefly, the surgical incision sites were shaved, cleaned, and sterilized. An incision was made in the left lower abdomen ~1 cm from the left leg and the femoral vein was exposed. A catheter (Micro-renathane tubing MRE-033-male; MRE-040-female, Braintree Scientific Inc., Braintree, MA) was inserted and passed subcutaneously to the scapulae where it was connected to the backplate (PlasticsOne, Roanoke, Virginia, USA, 8I313000BM14). The mesh of the backplate was sutured to the muscle, and then the skin closed around the backplate.

Spared Nerve Injury or Sham surgery

These surgeries were carried out as described in Decosterd & Woolf (2000). Briefly, the left femoral muscle was opened to expose the sciatic nerve. A 2 mm section of the peroneal and tibial nerves were removed, while the sural nerve was not disturbed. The exposed ends of the peroneal and tibial nerves were sutured to the underlying muscle to prevent regrowth. The sham surgery consisted of only the muscle incision, with no manipulation to any branch of the sciatic nerve. In both SNI and sham surgeries, the muscle was closed with absorbable suture, and the skin was closed with surgical staples (9mm; Braintree Scientific, Braintree, MA).

Apparatus

Self-administration experiments were conducted in 18 standard MED PC operant chambers (ENV-008CT, Med Associates, St. Albans, VT), which were housed in sound-attenuating chambers (ENV-018CT). These chambers were equipped with two nosepoke manipulandum on the right side of the chamber. A house light (white, ENV-114BM) was located on the top of the left side of the chamber. A variable speed pump (PHM-107) was used to deliver drug infusions. A 20 mL syringe was connected to the single channel, luer lock swivel (375/22PLS, Instech, Plymouth Meeting, PA) by luer lock tubing. Tygon tubing (100-80) was used to connect the swivel to the backplate, and the tygon tubing was contained in a stainless-steel spring. Data were collected via MED-PC software (SOF-735).

Procedures

Randall Selitto & Testing of Mechanical Hypersensitivity

To evaluate mechanical thresholds, a paw pressure applicator (Randall Selitto, IITC life sciences, Woodland hills CA) was used to apply continuously increasing amounts of pressure to the contralateral (non-surgerized limb) or ipsilateral (injured, sham injured) paws until the pressure caused a retraction of the hindpaw. The pressure resulting in hind paw retraction was recorded from the Analgesiometer with a 300 g pressure used as the maximum cutoff.

Prior to testing, rats were habituated to the hammock-like restraint over three days. On day one, rats were placed into the restraint for 5 min, and the habituation time was increased by 5 min/day until 15 min of habituation was achieved on the third day.

For all experiments, drugs were given i.v. and catheters were flushed with 0.5 mL heparinized saline daily for the duration of the experiment to maintain patency.

Self-Administration

Single component training sessions

Rats in self-administration experiments were allowed 7-10 days of post-operative recovery following catheter implant. All catheters were flushed daily during recovery with 0.5 mL 50 usp/mL heparinized saline. All self-administration sessions started with a catheter pre-fill infusion of (50 uL; confirm value). Rats were trained to self-administer fentanyl (0.0032 mg/kg/infusion) or cocaine (0.32 mg/kg/infusion) in daily 90 or 60 min sessions, respectively. Responding on the active nosepoke (illuminated) under a fixed ratio (FR)1 schedule of reinforcement resulted in delivery of drug, while responding on the inactive nosepoke had no scheduled consequences. After each infusion of drug, there was a 10 second time-out. The work requirement increased, across days, to a FR5 schedule of reinforcement. To proceed to the multi-dose procedure (details below), 5 consecutive days of stable responding were required. Stable responding was defined as no more than a 30% deviation in number of active responses (with no upward or downward trends) between days and <30% total responses occurred on the inactive nosepoke.

Multi-dose procedure

A multi-component, daily self-administration task was used to evaluate a range of doses of fentanyl *or* cocaine. Each session consisted of five, 25-min components with two-min blackouts between components during which responding had no scheduled consequence. For cocaine sessions: 0 (no infusion), 0.032, 0.1, 0.32, and 0.56 mg/kg cocaine were used across the components. For fentanyl sessions: 0 (no infusion), 0.32, 1, 3.2, 10, and 32 ug/kg/inf fentanyl were tested in overlapping dose response curves. Doses were presented in a semi-random order such

that the first component was always 0 mg/kg (no infusion) and the last component was always the largest dose tested (cocaine- 0.56 mg/kg/inf; fentanyl 10 or 32 ug/kg/inf). Components 2, 3, and 4 were randomized daily. Following 5 days of stable responding (same criteria as above), rats underwent either SNI or sham surgery. Following SNI or sham surgery, rats were allowed three days of post-operative recovery. Rats resumed self-administration on post-operative day four.

Drugs

Fentanyl citrate was obtained from Sigma Aldrich (Burlington, MA). Cocaine hydrochloride was obtained from NIDA Drug Supply. Ketamine hydrochloride was obtained from Hospira, INC (Lake Forest, IL). Xylazine was obtained from Hospira (Lake Forest, IL). Carprofen (Rimadyl) was obtained from (Zoetis, Parsippany, NJ). Fentanyl and cocaine were dissolved in sterile, physiological saline and delivered intravenously (i.v.). Xylazine was diluted from the stock solution to 20 mg/mL in sterile water. Carprofen was diluted to 5 mg/mL in sterile saline. Heparin was diluted to 50 usp/mL in sterile saline to produce heparinized saline. Xylazine and ketamine were delivered intraperitonially and carprofen was delivered subcutaneously.

Statistical Analysis

Data were analyzed by 3-way (dose, surgical status, time OR dose, surgical status, sex) or 4-way (dose, time, surgical status, sex) repeated measure ANOVAs in SPSS version 29. Significant main effects or interactions were followed up with one-way or two-way ANOVA's and post hoc analyses. Only main effects or interactions necessary for interpretation are reported. Corrections were made, if necessary, according to Mauchly's W criteria.

We intended to consider sex as a biological variable in all experiments. Unfortunately, we encountered difficulties such that we were unable to maintain catheter patency until the end of the experiment in female rats. Therefore, data are underpowered to detect sex differences. For Randall Selitto experiments, catheter patency was only needed for 9 days, and we were able to include sex as a biological variable.

Data in the graphs represent only animals that finished the procedure or at least progressed to 2 weeks following surgery.

Binned Data

For self-administration data, pre-surgical dose response curves were plotted as an average of the 5 stable days prior to surgery. Day four and day 9 dose effect curves are dose response curves from individual days. SNI/sham 2-week dose response curves reflect day 10-14 (days 4-9 removed for above analysis). SNI/sham 4-week dose response curves reflect days 24-28 (last 5 days of experiment) and this abbreviated time window is shown to match the other timepoints.

3.4 Results

Figure 3-1. Fentanyl self-administration prior to surgery and on post-operative days 4 and 9.

Figure 3-1A and D show fentanyl-maintained responses for each dose of fentanyl on post-operative days 4 and 9 in sham and SNI groups. There was a significant main effect of dose [$F(2.23, 31.51)=29.16$, $p<0.001$, $n2p=0.68$], suggesting that fentanyl maintained responding in a dose-dependent manner. Surprisingly, there was no main effect of time [$F(2, 28)=1.69$, $p=0.20$, $n2p=0.11$]. However, there was a significant interaction between dose and time [$F(2.96, 41.50)=0.52$, $p<0.001$, $n2p=0.65$], suggesting that fentanyl-maintained responding shifted over

time. This was likely due to the noticeable decrease in responding on day 4 in both sham and SNI groups as well as small differences in responding for 1 μg/kg fentanyl on day 9 as compared with pre-surgical dose effect curves.

There was no main effect: surgical status [$F(1, 14)=0.23$, $p=0.64$, $n2p=0.02$]. There were some changes in fentanyl-maintained responding; however, these changes are not due to surgical status [interaction: time* surgical status, $F(2, 28)=0.099$, $p=0.91$, $n2p=0.007$]. Further, there was no significant three-way interaction between time, dose, and surgical status [$F(2.96, 41.50)=0.52$, $p=0.67$, $n2p=0.04$]. Together, these data suggest that, although fentanyl-maintained responding was altered over time, surgical status did not explain these changes.

Figure 3-1B and E show the number of infusions of fentanyl earned for each dose of fentanyl on post-operative days 4 and 9. There was a significant main effect of dose [$F(1.94, 27.14)=75.05$, $p<0.001$, $n2p=0.84$], suggesting different numbers of infusions were earned per dose. The number of fentanyl infusions earned was altered over time, reflected by a significant main effect of time [$F(2,28)=20.27$, $p<0.001$, $n2p=0.59$]. Further, there was a significant interaction between dose and time [$F(6, 84)=4.40$, $p<0.001$, $n2p=0.24$], which likely reflects that the number of infusions earned was most different at lower doses of fentanyl.

Though fewer infusions of fentanyl were earned on day 4 post-surgery, this change was not explained by surgical status, supported by no main effect of surgical status [$F(1, 14)=0.23$, $p=0.64$, $n2p=0.02$] and no interaction between time and surgical status [$F(1, 14)=0.23$, $p=0.64$, $n2p=0.02$]. Additionally, there was no three way interaction between time, dose, and surgical status [$F(6, 84)=0.19$, $p=0.98$, $n2p=0.01$]. Collectively, these data suggest that the decrease in fentanyl infusions earned on post-operative day 4 was not explained by surgical status.

Figure 3-1C and E show the total fentanyl intake for each dose of fentanyl on post-operative days 4 and 9. Fentanyl intake was dose-dependent, reflected by a main effect of dose [$F(1.93, 26.98)=103.90$, $p<0.001$, $n2p=0.88$]. Fentanyl intake was altered over time, reflected by a main effect of time [$F(2, 28)=16.22$, $p<0.001$, $n2p=0.54$]. This was further supported by a significant time by dose interaction [$F(2.21, 84)=3.88$, $p=0.03$, $n2p=0.22$], which likely represents the decrease in fentanyl intake on post-operative day 4.

Though fentanyl intake was subject to changes over time, these changes were not explained by surgical status, supported by no main effect of surgical status [$F(1, 14)=0$, $p=0.998$, $n2p=0$] as well as no interaction of time and surgical status [$F(2, 28)=2.05$, $p=0.15$, $n2p=0.13$]. Lastly, there was no three-way interaction of time, surgical status, and dose [$F(6, 84)=1.40$, $p=0.23$, $n2p=0.09$]. Collectively, these results suggest that the decrease in fentanyl intake on post-operative day 4 was not explained by surgical status.

Figure 3-2. Fentanyl self-administration prior to surgery and over 2- to 4-weeks following surgery.

Figure 3-2 A and D show the fentanyl-maintained responding for each dose of fentanyl 2- or 4-weeks following surgery. Fentanyl maintained dose dependent responding, reflected by a main effect of dose [$F(2.15, 25.85)=71.05$, $p<0.001$, $n2p=0.86$]. There was no difference in fentanyl-maintained responding in SNI and sham groups, reflected by no main effect of surgical status [$F(1, 12)=3.68$, $p=0.08$, $n2p=0.24$] (Fig. 3-2A, D). There was no main effect of time such that fentanyl-maintained responding did not significantly change from pre- to post-surgical timepoints [$F(2, 20)=2.44$, p-0.11, $n2p=0.20$]. However, there was an interaction between dose and time [$F(8, 96)=2.40$, $p=0.02$, $n2p=0.17$], suggesting that there were small, but significant,

changes in responding for individual doses over time. For example, responding for 10 ug/kg/inf fentanyl at 4 weeks post SNI was increased as compared with pre-surgery in both sham and SNI groups. Additionally, responding for 1 ug/kg/inf decreased 2- or 4- weeks post SNI as compared with pre-surgery. However, there was no three-way interaction between surgical status, dose, and time [F(8, 96)=1.08, p=0.38, n2p=0.083]. All other interaction terms failed to reach significance (p's>0.12).

Figure Fig. 3-2 B and E show the number of fentanyl infusions earned for each dose of fentanyl 2- or 4-weeks following surgery. Prior to and after SNI or sham surgery, the number of fentanyl infusions earned per component was dose-dependent, reflected by a main effect of dose [doses included: 0.32-10 ug/kg, F(1.66, 21.62)=68.23, p<0.001, n2p=0.84]. There was no difference in fentanyl self-administration between sham and SNI animals suggesting that SNI-induced chronic neuropathic pain did not alter fentanyl self-administration, reflected by a no main effect of surgical status [F(1, 13)=0.41, p=0.46, n2p=0.04] (Fig. 3-2C, F).

There was a significant interaction between surgical status and time, suggesting there were some alterations in number of infusions earned over time in SNI and sham groups [F(2, 26)=4.12, p=0.03, n2p=0.24]. This likely reflects small differences between groups over time such as in the SNI group, infusions of 1 ug/kg dropped slightly at both post-surgical timepoints as compared with pre-surgery, while in the sham animals, infusions of this dose drop at 2-weeks and returned to pre-surgical levels at 4-weeks. However, there was no three-way interaction between time, dose, and surgical status, suggesting that there were minimal changes in specific doses by surgical status over time [F(6, 78)=1.87, p=0.10, n2p=0.13].

Figure 3-2 C and F show the total fentanyl intake for each dose of fentanyl 2- or 4-weeks following surgery. Fentanyl intake was dose dependent, reflected by a main effect of dose [F(4,

52)=189.02, p<0.001, n2p=0.94]. While there was no main effect of time [F(1.24, 12.36)=2.85, p=0.11, n2p=0.22], there was a significant interaction between time and dose, suggesting intake at specific doses changed over time [F(6, 78)=12.46, p<0.001, n2p=0.49]; for example, the intake of 10 ug/kg/inf fentanyl increased in the SNI group from the pre-surgical and 2-week timepoints to the 4-week timepoint. Lastly, there was no main effect of surgical status, indicating that fentanyl intake was not significantly different between sham and SNI groups [F(1, 13)=0.27, p=0.61, n2p=0.02]. Further, there was no interaction between surgical status and time [F(1.3, 16.89)=0.77, p=0.43, n2p=0.06], suggesting that intake of fentanyl was not changed over time by surgical status. All other interaction terms failed to reach significance (p's>0.12).

Figure 3-3. Cocaine self-administration prior to surgery and on post-operative days 4 and 9.

Figure 3-3 A and D show the cocaine-maintained responding for each dose cocaine on post-operative days 4 and 9. Cocaine maintained dose-dependent responding, supported by a main effect of dose [F(3, 18)=41.56, p<0.001, n2p=0.88]. Though there appeared to be a decrease in responding in both sham (A) and SNI (D) groups on day 4, this was not a significant from pre-surgical levels, supported by no main effect of time [F(1.53, 9.16)=0.19, p=0.78, n2p=0.03] as well as no interaction between time and dose [F(6, 36)=0.76, p=0.61, n2p=0.11]. Further, there was not no main effect of surgical status [F(1, 6)=0.007, p=0.94, n2p=0.001] and no interaction between time and surgical status [F(1.53, 9.16)=0.54, p=0.55, n2p=0.08]. Collectively these results demonstrated no significant change in cocaine-maintained responding over time or between SNI and sham groups.

Figure 3-3 B and E show the infusions earned for each dose of fentanyl tested on post-operative days 4 and 9. The number of infusions earned was dose-dependent, supported by a main

effect of dose [F(1.65, 9.91)=18.73, p<0.001, n2p=0.76]. While there was no main effect of time [F(2, 12)=0.13, p=0.88, n2p=0.02], there was a significant interaction between time and dose [F(6, 36)=2.69, p=0.03, n2p=0.31], suggesting that over time, there was a dose-dependent change in number of cocaine infusions. This likely reflects the decrease in infusions earned on post-operative day 4 in both sham (B) and SNI (E) groups.

While number of cocaine infusions earned was subject to dose-dependent changes over time, these changes were not explained by surgical status. This was supported by no main effect of time [F(2, 12)=0.47, p=0.64, n2p=0.07] and no interaction between time and surgical status [F(2, 12)=0.47, p=0.64, n2p=0.07]. Collectively, these results suggest that surgical status did not explain differences in cocaine infusions over time.

Figure 3-3 C and F show cocaine intake for each dose of cocaine on post-operative days 4 and 9. Cocaine intake was dose-dependent, reflected by a main effect of dose [F(1.78, 10.71)=120.08, p<0.001, n2p=0.95]. Cocaine intake was subject to changes over time, supported by a main effect of time [F(1.43, 8.60)=7.51, p=0.02, n2p=0.56], and these changes were most apparent at larger doses of cocaine (0.1-0.56 mg/kg/inf). This was supported by a significant interaction between time and dose [F(6, 36)=4.58, p=0.002, n2p=0.43].

While cocaine intake was subject to changes over time, these changes were not explained by surgical status [no main effect, surgical status, F(1, 6)=0.13, p=0.73, n2p=0.02]. this was further supported by no interaction between time and surgical status [F(1.43, 8.60)=0.38, p=0.63, n2p=0.06]. Collectively, these results suggest that cocaine intake was subject to small, significant shifts over time that were not explained by surgical status.

Figure 3-4. Cocaine self-administration prior to surgery and over 2- or 4-weeks after surgery.

Figure 3-4 A and D show cocaine-maintained responding for each dose of cocaine prior to and over 2- or 4-weeks following surgery. Prior to and after surgery, cocaine maintained responding in a dose-dependent manner, reflected by a main effect of dose [doses included: 0.032-0.56 mg/kg $F(2.17, 15.17)=80.95$, $p<0.001$, $n2p=0.92$]. Cocaine-maintained responding was not altered by up to 4 weeks of SNI or sham states, reflected by no main effect of time [$F(2, 14)=0.01$, $p=0.99$, $n2p=0.001$]. There was also no difference in cocaine-maintained responding between SNI and sham groups, reflected by no main effect of surgical status [$F(1, 7)=0.79$, $p=0.41$, $n2p=0.10$]. Further, there was no interaction between time and dose for this dataset [$F(8, 56)=0.86$, $p=0.55$, $n2p=0.11$], and there was no interaction between time, dose, and surgical status [$F(8, 56)=0.21$, $p=0.99$, $n2p=0.03$]. All other interaction terms failed to reach significance as well (p's>0.38).

Figure 3-4 B and E show the number of cocaine infusions earned in each component of the multidose procedure 2- or 4-weeks following surgery. The number of cocaine infusions was dose dependent, reflected by a main effect of dose [$F(4, 28)=80.95$, $p<0.001$, $n2p=0.92$]. There was no main effect of surgical status suggesting that there was no difference in number of infusions self-administered between sham and SNI groups [no main effect, surgical status: $F(1,7)=0.79$, $p=0.41$, $n2p=0.10$]. Further, there was no main effect of time, suggesting cocaine infusions were unaltered over the course of 4 weeks of SNI or sham states [$F(2, 14)=0.01$, $p=0.99$, $n2p=0.001$]. There also was not an interaction between time and dose [$F(8, 56)=0.86$, $p=0.55$, $n2p=0.11$] or time, dose, and surgical status [$F(8, 56)=0.21$, $p=0.99$, $n2p=0.03$]. All other interaction terms failed to reach significance (p's>0.39).

Figure 3-4C and F show the total cocaine intake in each component of the multidose procedure 2- or 4-weeks following surgery. Prior to surgery, cocaine intake increased as dose increased, through 0.32 mg/kg infusion, while intake decreased at the 0.56 mg/kg/inf dose,

reflected by a significant main effect of dose [F(1, 9)=4, 36)=237.19, p<0.001, n2p=0.96]. There was no difference in cocaine intake between SNI and sham groups, reflected by no main effect of surgical status [F(1, 9)=0.63, p=0.63, n2p=0.03]. Cocaine intake was not altered by up to four weeks of SNI or sham states, reflected by no main effect of time [F(2, 18)=0.042, p=0.96, n2p=0.005]. Further, there was no interaction between dose and time [F(8, 72)=0.25, p=0.98, n2p=0.03] or dose, time, and surgical status [F(8, 72)=1.61, p=0.14, n2p=0.15]. All other interaction terms failed to reach significance (p's>0.14).

Figure 3-5. Antihyperalgesic- and antinociceptive-like effects of intravenous fentanyl or cocaine in the Randall Selitto Assay.

The data shown in Figure 3-5A and B demonstrate that i.v. fentanyl produced dose-dependent antinociceptive-like and antihyperalgesic-like effects on days 4 and 9 following surgery. We evaluated 0.32-32 μg/kg fentanyl as these are the doses available following surgery. SNI surgery induced a hyperalgesic-like state, as demonstrated by lowered baseline paw withdrawal thresholds, reflected by a main effect of surgical status [F(1, 20)=2.97, p<0.001, n2p=0.65]. There was a significant main effect of dose such that fentanyl produced dose dependent increases in paw withdrawal thresholds [F(2.9, 58.07)=495.69, p<0.001, n2p=0.96]. Additionally, there was a dose by surgical status interaction [F(2.90, 58.07)=19.76, p<0.001, n2p=0.50]. This reflects lower baseline paw withdrawal thresholds in the SNI group with no fentanyl on board and low dose fentanyl (0.32 and 1 ug/kg). A one-way ANOVA split by surgical status and Tukey's post hoc analyses to determine which doses of fentanyl significantly increased paw withdrawal thresholds compared to vehicle in both sham and SNI groups. There was a main effect of dose in both sham [F(5, 66)=116.73, p<0.001, n2=0.90] and SNI groups [F(5, 66)=212.84, p<0.001,

n2=0.94]. In sham rats, 3.2-32 ug/kg fentanyl significantly increased paw withdrawal thresholds as compared with vehicle (all p<0.001). In SNI rats, 1 (p=0.003), 3.2 (p<0.001), 10 (p<0.001), and 32 (p<0.001) ug/kg fentanyl significantly increased paw withdrawal thresholds as compared with vehicle.

Fentanyl was approximately equally effective on day 4 and 9 following surgery, as reflected by a non-significant main effect of time [F(1, 20)=1.19, p=0.29, n2p=0.06] as well as a no interaction between time and dose [F(5, 100)=1.63, p=0.16, n2p=0.08]. There was no sex difference in fentanyl-induced antinociceptive- or antihyperalgesic-like effects [F(1, 20)=0.08, p=0.78, n2p=0.004]. All other interaction terms failed to reach significance (p's<0.1).

As shown in Figure 3-5C and D, i.v. fentanyl produced dose dependent antinociceptive- and antihyperalgesic-like effects 2.5 months after injury. SNI surgery induced a hyperalgesic-like state as demonstrated by lowered baseline paw withdrawal thresholds as compared with sham, reflected by a main effect of surgical status [F(1, 20)=51.62, p<0.001, n2p=0.72]. There was a significant main effect of dose such that fentanyl produced dose dependent increases in paw withdrawal thresholds [F(2.88, 57.63)=278.03, p<0.001, n2p=0.93]. A one-way ANOVA split by surgical status and Tukey's post hoc analyses was used to determine which doses of fentanyl significantly increased paw withdrawal thresholds compared to vehicle in both sham and SNI groups. There was a significant main effect of dose in sham groups [F(5, 66)=118.67, p<0.001, n2=0.9] and SNI groups [F(5, 66)=105.52, p<0.001, n2=0.89]. 3.2-32 ug/kg fentanyl were significantly more effective than vehicle in sham (all p<0.001) and SNI groups (all p<0.001).

There was no main effect of sex [main effect: sex, [F(1, 20)=1.64, p=0.22, n2p=0.08]. Though, there was an interaction between dose and sex, suggesting differences in the fentanyl dose effect curves by sex [F(2.88, 57.63)=4.88, p=0.005, n2p=0.20]. This likely represents the slightly

increased effectiveness of 1 and 3.2 ug/kg in males (Fig. 3-5C). All other interactions failed to reach significance, including the three-way interaction term (p's>0.86).

We tested 0.56 mg/kg cocaine as it is the highest dose available in self-administration assays, and 0.56 mg/kg was tested as a time course. As shown in Figure 3-5E and F, i.v. cocaine (0.56 mg/kg) failed to attenuate SNI-induced mechanical hypersensitivity in either sex. 0.56 mg/kg cocaine (i.v.) failed to alter paw withdrawal over a 10 min period in males (Fig. 3-5E) and females (Fig. 3-5F), reflected by a non-significant main effect of time [F(4.12, 82.33)=2.04, p=0.10, n2p=0.09]. There was significant difference between sham and SNI groups such that, at all points tested, SNI groups had lower paw withdrawal thresholds than sham groups [main effect: surgical status, F(1, 20)=132.82, p<0.001, n2p=0.87]. Lastly, there was no main effect of sex [main effect: sex, F(1, 20)=1.16, p=0.29, n2p=0.06], indicating a lack of sex differences. All interaction terms failed to reach significance (p's>0.17)

3.5 Discussion

The goal of the present study was to evaluate if SNI-induced hypersensitivity altered ongoing self-administration of fentanyl or cocaine. To determine if SNI-induced hypersensitivity altered ongoing fentanyl or cocaine self-administration, animals were first trained to self-administer fentanyl *or* cocaine, then underwent surgery, resumed self-administration on post-operative day 4, and post-operative evaluation of fentanyl-maintained behavior continued for 4 weeks. At all timepoints, the fentanyl dose effect curves were examined across three variables (i.e., responding, infusions, intake). Prior to surgery, fentanyl-maintained responding and intake dose-dependently increased (Fig. 3-1). Compared with fentanyl dose effect curves established prior to surgery, fentanyl-maintained behavior was significantly decreased on post-operative day 4 in both

sham and SNI groups (Fig. 3-1A, D). However, this decrease in fentanyl self-administration did not persist over the remaining duration of post-operative evaluation. By post-operative day 9, fentanyl self-administration behaviors returned to approximately pre-surgical levels in both sham and SNI groups (Fig. 3-1). Decreases in fentanyl self-administration were observed in both sham and SNI groups, suggesting SNI-induced hypersensitivity does not explain the observed decreases in fentanyl self-administration. While sham surgery does not include damage to the sciatic nerve, a large muscle incision is made. Although the sham groups received carprofen to manage post-operative pain and/or discomfort, post-operative pain or surgery itself and exposure to anesthesia may contribute to the decrease in fentanyl self-administration immediately after surgery. Decreased self-administration in sham groups has been observed before in other studies (Wade et al., 2003).

To evaluate if fentanyl effectively alleviates hypersensitivity to mechanical stimulation on post-operative day 4, a separate group of rats was used to evaluate the effects of intravenous fentanyl on paw withdrawal thresholds. Paw withdrawal thresholds following vehicle administration were similar to thresholds observed in naïve rats and to threshold measured prior to surgery (see Ch. 2 Figures 2-1B, 2-3). Together, these data suggest that the sham groups were not hypersensitive to mechanical stimulation 4- or 9-days post-surgery. However, it is somewhat challenging to completely dismiss post-operative pain as an explanation for the initial decrease in fentanyl maintained-responding as there is a large difference in the environment between Randall Selitto assays (i.e., animals suspended from hammock-like restraint with no weight on hind paws) and the self-administration chambers (i.e., animals support full body weight, move on grid floors).

Fentanyl self-administration behaviors were examined over a total of 4 weeks following surgery in order to determine if chronic pain states alter the ongoing self-administration of MOR

agonists over an extended period of time. The initial decrease in fentanyl self-administration was not persistent, such that, at 2- or 4-weeks following surgery, there were no robust shifts in the fentanyl dose effect curves. Nevertheless, there was evidence of increased intake of specifically 10 ug/kg fentanyl in both SNI and sham groups (Fig. 3-3). This finding is consistent with decreased sensitivity, or tolerance development, to MOR agonist-induced effects following repeated self-administration (Dao et al., 2021; Malone et al., 2021; Martyn et al., 2019).

It is also possible that tolerance developed to the rate suppressant effects of fentanyl (rather than the reinforcing effects), such that animals experience less behavioral disruption or sedation at larger doses over time and, therefore, intake of large fentanyl doses could increase. Repeated administration of MOR agonists has been previously demonstrated to produce rightward shifts in the opioid analgesic-induced locomotor suppressing effects (Timar, Gyarmati, & Furst, 2008). Overall, in the present study, while there were changes in fentanyl intake over time, SNI-induced hypersensitivity failed to alter the ongoing self-administration of fentanyl. This finding is consistent with a couple of previous studies in which acute or sub-chronic pain states did not alter ongoing opioid self-administration (Barattini et al., 2023; Reiner et al., 2021).

However, several other studies report an increase or decrease in the self-administration of MOR agonists in the presence of a pain state (Table 3-1, 3-2). While there are many methodological differences in the present and previous studies, two factors may contribute to the differential effects of pain on opioid self-administration across studies: (1) when pain states were induced (prior to or after acquisition of self-administration, Table 3-1) and (2) the type and duration of pain used (Table 3-2). As shown in Table 3-1, a large number of previous studies have induced pain states prior to acquisition of MOR agonist self-administration while relatively few have induced pain after opioid self-administration was established. The type of pain utilized (e.g.,

neuropathic or inflammatory) may differentially alter the reinforcing effects of MOR agonists (Table 2). Only one previous study has examined if chronic neuropathic pain alters ongoing self-administration of MOR agonists, and this study demonstrated that in male mice, paclitaxel administration prevented the decrease in morphine breakpoint over time, compared to the sham group (Neelakanten et al., 2017). These findings suggest a very small impact of paclitaxel on morphine-induced reinforcing effects.

Overall, the primary goal of the present study was to determine if chronic neuropathic pain or sham states altered the ongoing self-administration of fentanyl, but we also evaluated if pain states alter the reinforcing effects of non-opioidergic drugs of abuse, which no previous studies have tested. Collectively, the present study demonstrated that SNI-induced hypersensitivity did not alter the reinforcing effects of fentanyl or cocaine. Though, we observed changes in fentanyl induced reinforcing effects over time, such that animals in both sham and SNI groups responded more for 10 ug/kg/inf fentanyl, SNI-induced hypersensitivity did not explain the increased intake of 10 ug/kg/inf fentanyl.

The failure of SNI-induced hypersensitivity to alter reinforcing effects or antinociceptive- and antihyperalgesic-like effects of fentanyl could be due to use of a high efficacy MOR agonist in these experiments. Previous studies have reported that pain states generally reduce MOR expression and activity in reward circuitry (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Ozaki et al., 2002; Pol et al., 2006; Porecca et al., 1998; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998); however, it is possible these changes in receptor expression or activity may not be sufficient to alter behavioral effects of a high efficacy agonist. Future studies should examine the reinforcing effects of a lower efficacy MOR agonist such as nalbuphine or buprenorphine.

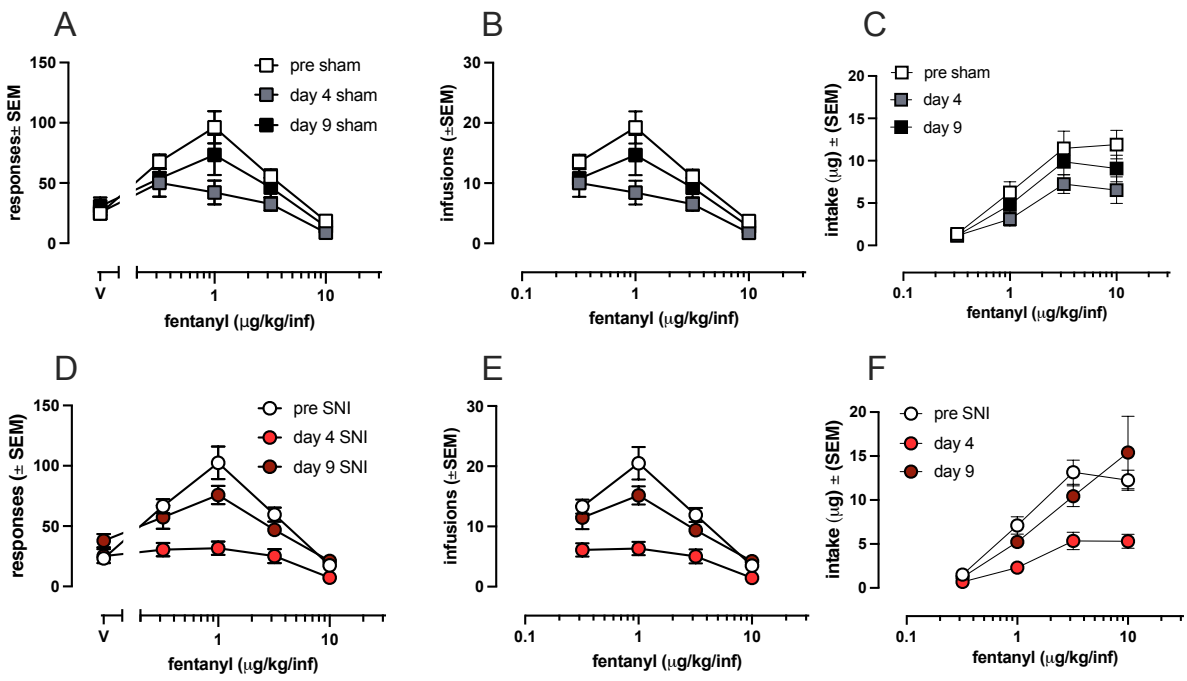

Figure 3-1. Self-administration of fentanyl prior to surgery and on post-operative days 4, 9

Data are plotted as responses for fentanyl (A, D), infusions of fentanyl earned (B, E), and total intake (μg) of fentanyl (C, F). Fentanyl maintained self-administration in all rats under a fixed ratio (FR)5 schedule of reinforcement prior to surgery. On post-operative day 4, there was a significant reduction in fentanyl self-administration in both sham (A-C) and SNI (D-F) groups; however, by day 9 following surgery there was no significant difference in fentanyl maintained responding compared to pre-surgical dose effect curves. Data are plotted as the mean ± SEM. Each data point represents 8-10 rats and include both males and females.

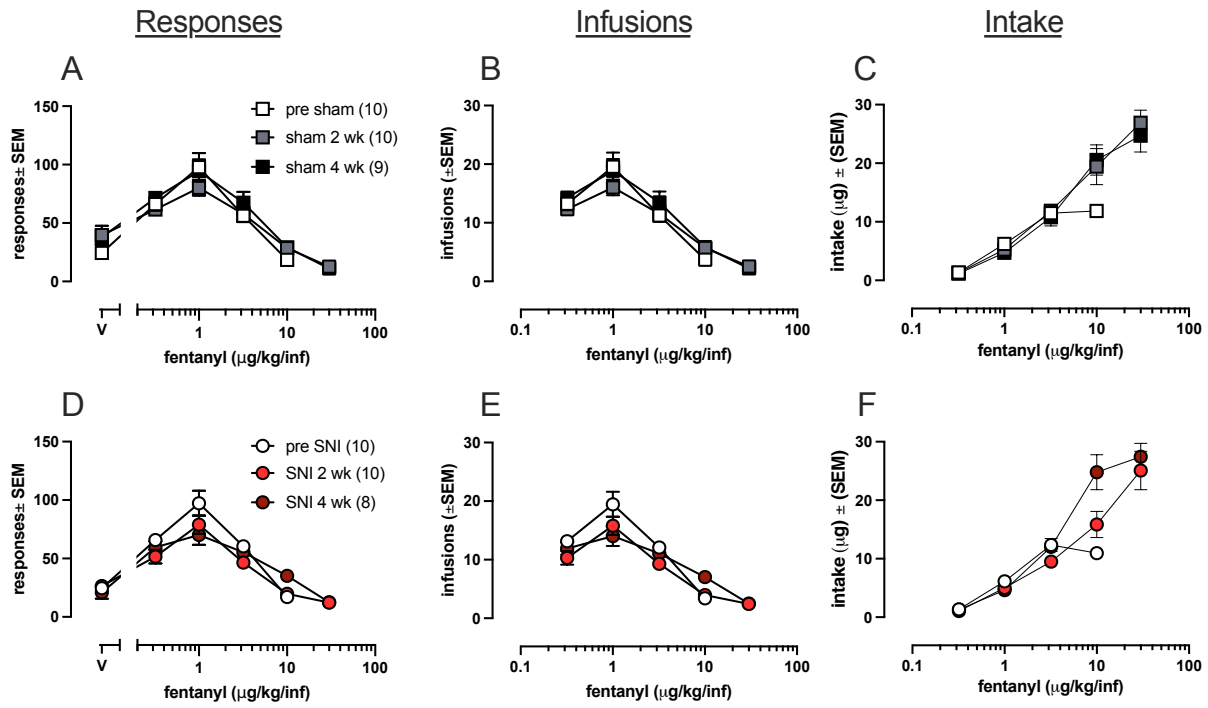

Figure 3-2. Self-administration of fentanyl prior to and after sham or SNI surgery

Data are plotted as responses for fentanyl (A, D), infusions of fentanyl earned (B, E), and total intake (µg) of fentanyl (C, F). Fentanyl maintained self-administration in all rats under a fixed ratio (FR)5 schedule of reinforcement prior to surgery (pre-surgical dose effect curves are the same as plotted in Figure 1). There was no significant difference between fentanyl-maintained responding 2- or 4- weeks following sham (A-C) or SNI (D-F) surgery. After surgery, both sham and SNI groups had increased intake of 10 *u*g/kg fentanyl (C, F). Data are plotted as the mean ± SEM. Each data point represents 8-10 rats and include both males and females.

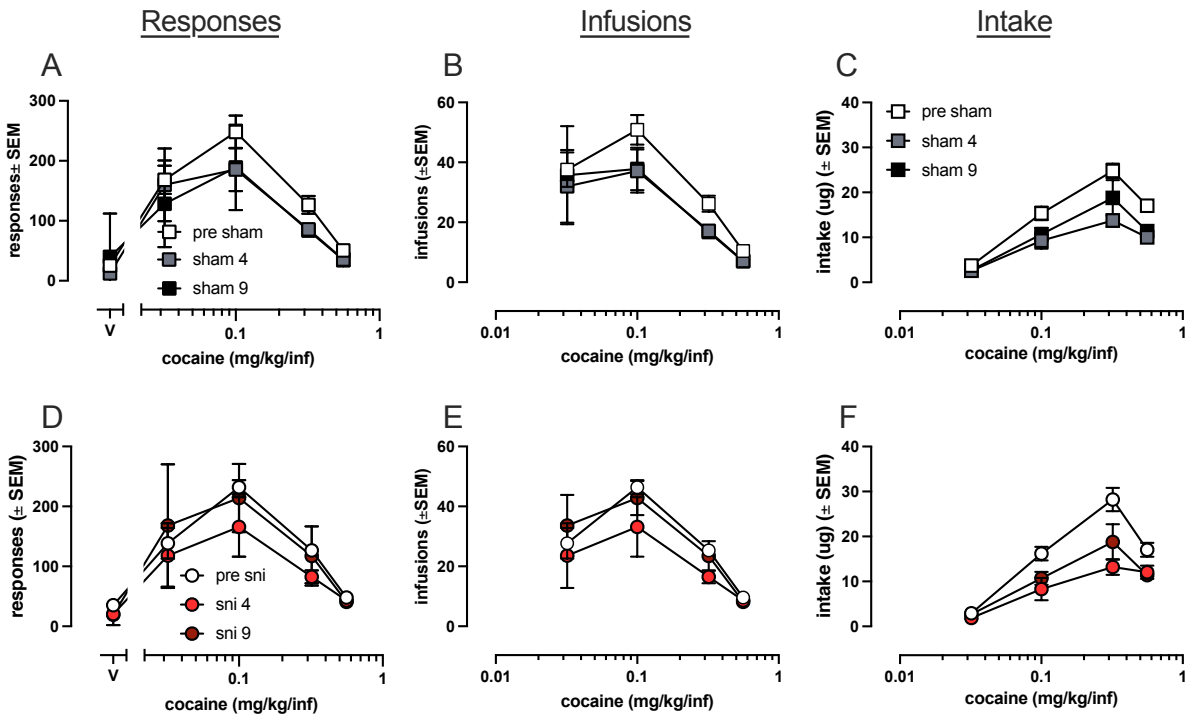

Figure 3-3. Self-administration of cocaine prior to surgery and on post-operative days 4, 9

Data are plotted as responses for cocaine (A, D), infusions of cocaine earned (B, E), and total intake (μg) of cocaine (C, F). Cocaine maintained self-administration in all rats under a fixed ratio (FR)5 schedule of reinforcement prior to surgery. There was no significant difference in cocaine self-administration on post-operative days 4 and 9 compared to pre-surgical dose effect curves in either sham (A-C) or SNI (D-F) groups. Data are plotted as the mean ± SEM. Each data point represents 8-10 rats and include both males and females.

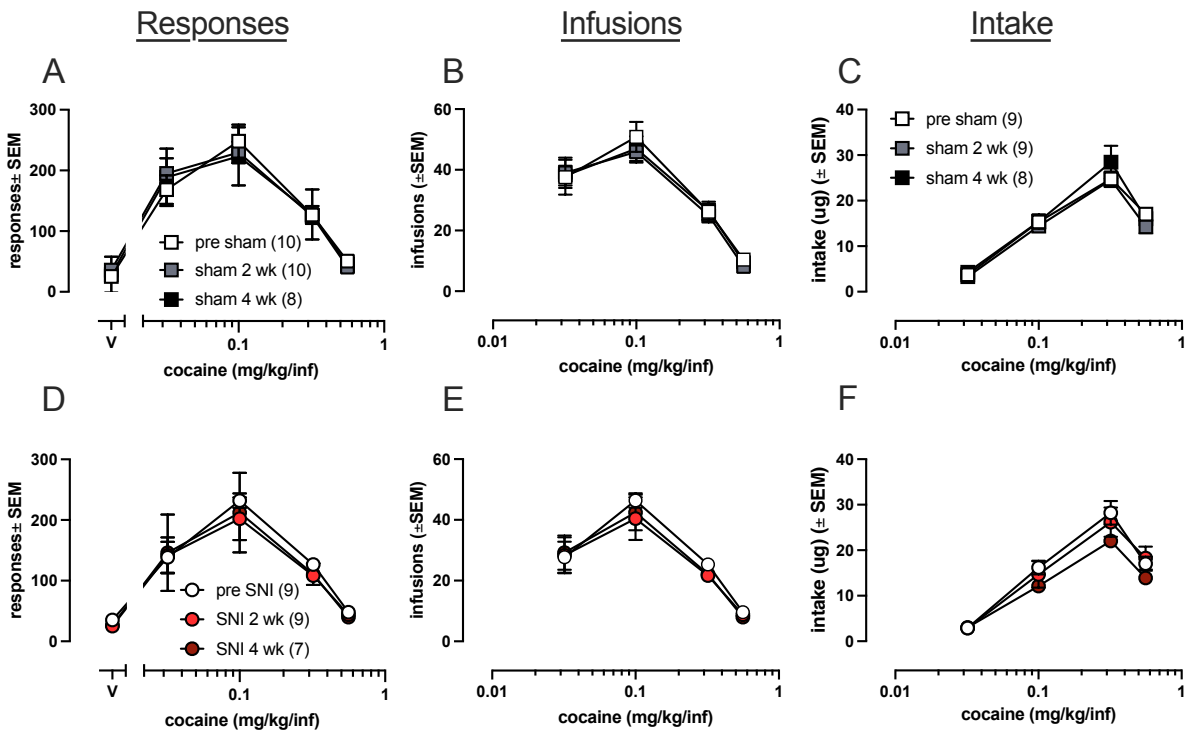

Figure 3-4. Figure 3-5. Self-administration of cocaine prior to and after surgery

Data are plotted as responses for cocaine (A, D), infusions of cocaine earned (B, E), and total intake (μg) of cocaine (C, F). Cocaine maintained self-administration in all rats under a fixed ratio (FR)5 schedule of reinforcement prior to surgery. There was no significant difference in cocaine self-administration 2- or 4- weeks following either sham (A-C) or SNI surgery (D-F). Data are plotted as the mean ± SEM. Each data point represents 8-10 rats and include both males and females.

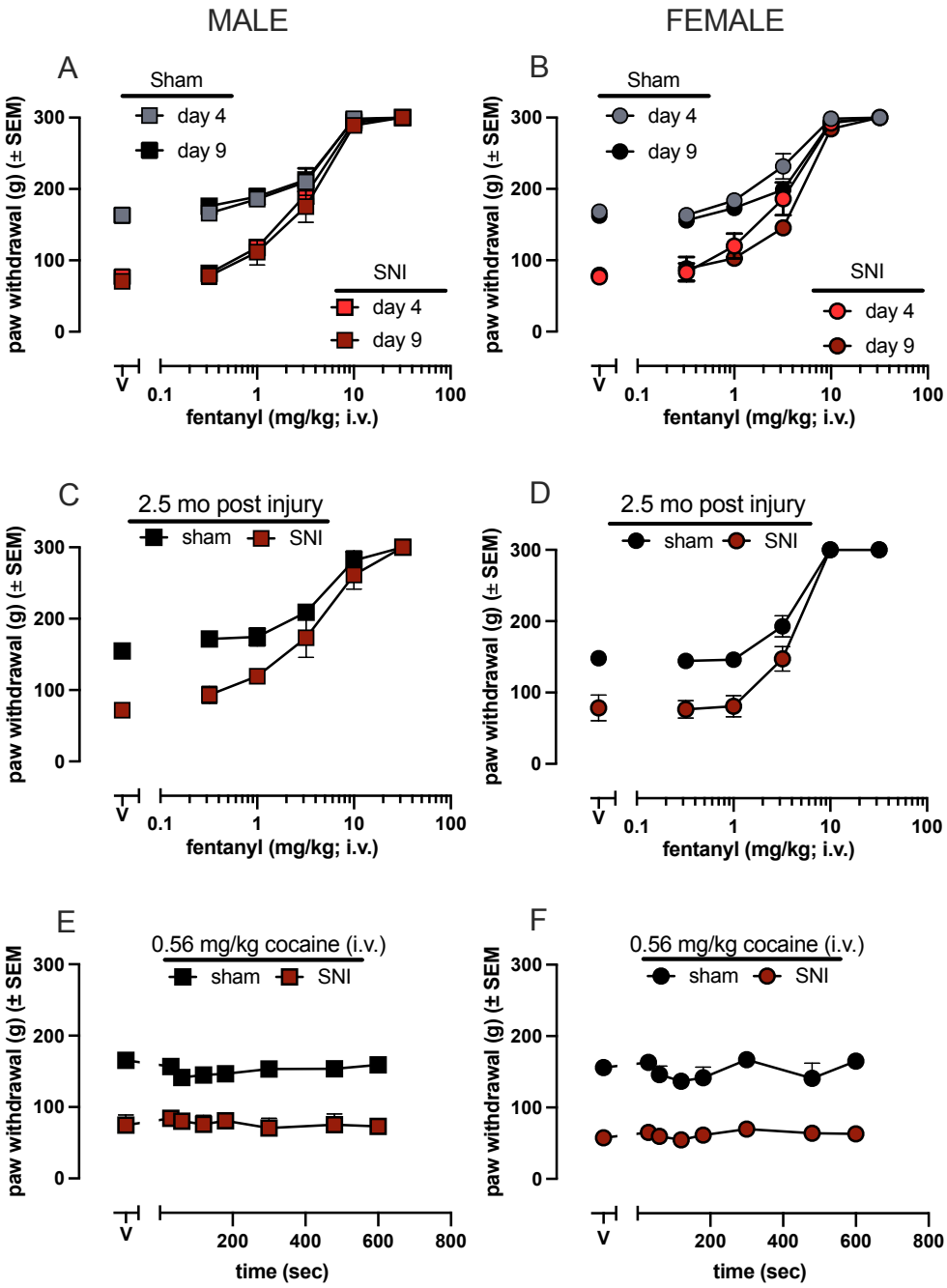

Figure 3-5. Effect of intravenous fentanyl or cocaine on paw withdrawal thresholds

Effects of intravenous fentanyl (0-32 µg/kg) or cocaine (0.56 mg/kg) on paw withdrawal thresholds in male and female rats. Fentanyl induced antinociceptive- and antihyperalgesic-like effects were evaluated on post-operative days 4 and 9 (A, B) as well as 2.5 months following surgery (C, D). Cocaine-induced alterations in paw withdrawal thresholds were evaluated once, as

a time course (E, F). Data are plotted as the mean (± SEM) paw withdrawal threshold. Each datapoint represents the average of 6 rats.

Previous studies investigating impact of pain states on MOR agonist-maintained behavior

Time Pain Induced					
Before Acquisition			After Acquisition		
Decrease	No change	Increase	Decrease	No change	Increase
Martin et al., 2007	Hou et al., 2015	Higginbotham et al., 2023	N/A	Reiner et al., 2021 (	Dib & Duclaux, 1982
Lyness et al., 1989	-	Hipolitto et al., 2015	-	Barattini et al., 2023	-
Woller et al., 2014	-	Colpaert et al., 2001	-	Neelakanten et al., 2017	-
Wade et al., 2003	-	-	-	Present Study	-

Table 3-1. Previous studies categorized by the time pain was induced

Previous studies examining effect of pain states on self-administration behavior sorted by time pain state introduced.

Previous studies investigating impact of pain states on MOR agonist-maintained behavior

Type of Pain					
Neuropathic			**Inflammatory, nociceptive**		
Decrease	No change	Increase	Decrease	No change	Increase
Wade et al., 2023 (SNL*, chemotherapeutic induced neuropathy#)	Neelakanten et al., 2017 (chemotherapeutic induced neuropathy)l	N/A	Wade et al., 2023 (CFA#)	Reiner et al., 2021 (lactic acid^, capsaicin^)	Dib & Duclaux, 1982 (nociceptive stimulation-shock^)
Martin et al, 2007 (SNL*)	Present study	-	Lyness et al., 1989 (adjuvant arthritis*)	Baratinni et al., 2023 (CFA#)	Colpaert et al., 2001 (adjuvant arthritis*)
-	-	-	-	Hou et al., 2015 (CFA#)	Higginbotham et al. 2023 (CFA#)
-	-	-	-	-	Hipolitto et al., 2015 (CFA#)

Table 3-2. Previous studies sorted by the type (and duration) of pain state

Previous studies examining effect of pain states on self-administration behavior sorted by type of pain state used (inflammatory, neuropathic) Symbols refer to duration of pain state such that * = chronic, # = sub-chronic, and ^ = acute.

Chapter 4
The Effects of Chronic Neuropathic Pain States on the Discriminative Stimulus Effect of Fentanyl and other MOR Agonists

4.1 Abstract

The subjective effects of drugs have been demonstrated to contribute to abuse potential. In humans, exposure to temporary noxious stimuli has been reported to decrease the pleasant subjective effects of mu opioid receptor (MOR) agonists, suggesting that chronic pain may alter the abuse potential of MOR agonists. However, no studies have evaluated if chronic pain alters the discriminative stimulus of MOR agonists. Therefore, the goal of this study was to evaluate the discriminative stimulus of fentanyl in the presence or absence of chronic neuropathic pain. Animals were trained to discriminate 0.032 mg/kg fentanyl from saline, and pre-surgical testing of morphine or nalbuphine generalization to the fentanyl discriminative stimulus were evaluated. Then, animals underwent sham or spared nerve injury (SNI) surgery, were allowed 72 hr of post-operative recovery, and the fentanyl discriminative stimulus was repeatedly evaluated for 4 months of sham or SNI states. Fentanyl produced dose dependent increases in % fentanyl responding prior to either surgery, and sham and SNI states failed to induce shifts the fentanyl dose effect curve over 4 months. Morphine dose-dependently increased % fentanyl responding prior to surgery and after sham or SNI surgery with minimal changes over 4 months. Nalbuphine dose-dependently increased % fentanyl responding prior to surgery; however, following either sham or SNI surgery, nalbuphine was more potent. Collectively, these results suggest that the fentanyl discriminative

stimulus is not altered by the presence of chronic neuropathic pain; however, there were observed small shifts in the dose response curves in fentanyl, morphine, and nalbuphine induced effects over time. A separate group of animals was trained to discriminate 5.6 mg/kg cocaine (i.p.) from saline prior to the induction of sham or SNI states, and cocaine dose-dependently increased % cocaine responding. Following either sham or SNI surgery, there was no change in the cocaine discriminative stimulus; however, quinpirole induced higher levels of % cocaine responding in SNI animals. This was observed to a lesser extent in the sham animals as well. Collectively, these changes suggest that the abuse potential of fentanyl or cocaine is not altered by the presence of chronic neuropathic pain; however, future studies should directly examine the abuse potential of dopaminergic agonists in the presence or absence of chronic neuropathic pain states.

4.2 Introduction

Opioid analgesics are known to produce both pleasant (e.g., "euphoric"), and unpleasant (e.g., "dizzy", "nauseous") subjective effects in humans (Comer et al., 2008; 2009; 2010; Lasagna et al., 1955; Zacny, 2001). In non-human animals (non-verbal), a correlate of subjective effects is measured through use of drug discrimination assays, and there is generally a similarity in observed drug effects across species (Schuester & Johnasen, 1976; Riley et al. 2016). While the internal state produced by drug may not be evident to others, these private, internal, subjective effects can serve as discriminative stimuli and exert discriminative control over behavior once animals are trained. The discriminative stimulus effects of opioid analgesics, or mu-opioid receptor (MOR) agonists, have been well-characterized in rodents, monkeys, and humans (for example: rodent, Colpaert & Janssen, 1986; Pournaghash & Riley, 1993; Shannon & Holtzman, 1976; Walker & Young, 1993; monkey: DeRosset & Holtzman, 1986; France, 1994; Negus, Picker, & Dykstra,

1991, human: Comer et al., 2008; 2009; 2010; Lasagna et al., 1955; Zacny, 2001), and all MOR agonists share similar discriminative stimulus properties. For example, in rats trained to discriminate an injection of morphine from saline, drugs with lower intrinsic efficacy (e.g., buprenorphine) partially generalize to the discriminative stimulus effects of morphine (Jones, Bigelow, & Preston, 1999; Brandt et al., 1997). However, compounds from other drug classes (e.g., pentobarbital) should not produce morphine-like responding (Broadbent et al., 1995; Colpaert, 1999). Importantly, drug discrimination assays can also be used to evaluate pharmacological phenomena, such as tolerance and dependence (Colpaert et al., 1976; Young, Kapitsopoulos, & Makhay, 1991; Paronis & Holtzman, 1985), and to dissect the overlapping mechanisms produce by compound pharmacological cues.

The discriminative stimulus effects of opioid analgesics may also be altered by acute and chronic pain. For example, several studies in humans have demonstrated that exposure to escapable, noxious stimuli (e.g., cold pressor pain) decrease reported pleasant subjective effects of prescription opioid analgesics (Comer et al., 2010; Zacny & Bekman, 2004), but we know relatively little about if chronic, ongoing/inescapable pain alters the subjective effects of MOR agonists. Similarly, in mice, acute administration of a chemical noxious stimulus (0.4% acetic acid, i.p.) produced a ~2.2 fold shift in the morphine dose effect curve in male, but not female, mice (Neelakanten, et al., 2015). These data collectively suggest exposure to a noxious stimulus may alter the interoceptive effects of MOR agonists. However, no studies have examined how ongoing, chronic pain states may alter the discriminative stimuli of MOR agonists.

While opioid analgesics are not generally warranted for treatment of chronic pain, recent data suggest that more than half (~69%) chronic neuropathic pain patients receive opioid analgesic treatment. Therefore, the goal of this study was to assess the extent to which a long-lasting pain

state altered the discrimination of a MOR agonist (fentanyl) from saline or a non-opioid drug of abuse (cocaine) from saline in both male and female rats. In order to induce chronic pain-like states in rodents, we utilized the spared nerve injury (SNI) model developed by Decosterd & Wolfe (2000); this model was chosen for the incredibly persistent hyperalgesic-like state induced. Prior studies have demonstrated this state lasts at least 8 months following injury (Erichsen & Blackburn-Munro, 2008).

Methods

Animals

Female and male Sprague-Dawley rats were purchased from Envigo labs (Indianapolis, IN). Following arrival, rats were group-housed and allowed at least one week of acclimation. All animals were housed in clear plastic cages with corncob bedding. Animal housing facilities were maintained on 12 hour light:dark cycle with lights on at 07:00. Seven to-10 days after arrival, rats were single housed for 3-4 days with food and water available ad libitum. Rats were at least eight weeks old at the start of experiments. Approximately 72 hours prior to initiation of operant training, rats were single-housed, food restricted with males given ~18 grams and females given ~14 grams standard rat chow (5LOD, LabDiet, Tuscon, AZ) daily, and given 20 sucrose pellets (45 mg pellets, unflavored; F0023; Bioserv, Flemmington NJ) in their home cage.

Surgery

The spared nerve injury (SNI) or sham surgeries were performed as described by Decosterd & Wolfe (2000). Briefly, rats were anesthetized with 90 mg/kg ketamine (i.p.) and 10 mg/kg xylazine (i.p.). Carprofen (5 mg/kg, s.c.) was given prior to surgery as pre-operative analgesia as

well as 24- and 48 hr after surgery for post-operative analgesia. An incision was made in the left femoral muscle to expose the sciatic nerve. In the SNI condition, a 2 mm portion of the tibial and peroneal branches was removed while the sural branch was left unmanipulated. In the sham condition, the femoral muscle incision was made, but there was no manipulation of any branch of the sciatic nerve. In both surgeries, the muscle and skin incision were closed with absorbable suture and surgical staples, respectively.

Apparatus

All experiments were conducted in 18 standard Med PC operant chambers (ENV-008CT, Med Associates, St. Albans, VT) housed inside ventilated sound-attenuating chambers (ENV-018CT). Chambers were equipped with two illuminated nosepoke devices (ENV-114BM), a food hopper, a pellet dispenser, a white house light, and three panel stimulus lights. Nosepoke manipulandum were located on the right side of the chamber on either side of the pellet hopper (ENV-200R2M). Three panel LED stimulus lights were located above both nosepokes (ENV-114BM). The pellet hopper was connected to a pellet dispenser (ENV-203-45) filled with 45 mg dustless, unflavored sucrose pellets (F0023, Bioserv, Flemmington, NJ). A white house light was located on the left side of the chamber.

Procedure

Operant training

Training, testing, and maintenance sessions were modeled on previous work (Jutkiewicz et al., 2011). Illumination of the lights in the nosepokes signaled the initiation of response periods. First, animals were trained to respond for 45 mg sucrose pellets in a 20-min session in which

responding on either nosepoke resulted in sucrose pellet delivery and illumination of the house light. The response requirement was gradually increased from fixed ratio (FR)1 to FR10 over time. was assessed in each rat. Next, a 5-min blackout period was introduced at the beginning of the session, which increased to 15 min over 6-8 sessions. Simultaneously, the responding period was decreased from 20 min to 5 min over 5-10 sessions. Therefore, a single component was comprised of a 15 min blackout and 5 min response (S^D) period. At this time, individual preferences of left or right nosepoke manipulanda were determined.

Discrimination Training

Training drug dose was assigned to the preferred nosepoke in ~50% of rats. Rats were trained to discriminate 0.032 mg/kg fentanyl (s.c.) from saline or 5.6 mg/kg cocaine (i.p.) from saline. Saline or training drugs were administered by the experimenter immediately prior to the start of each component. Following 15 min of blackout, responding on a FR10 schedule of reinforcement on the injection-appropriate nosepoke was reinforced with 45 mg sucrose pellet delivery during the 5-min S^D period o. Each pellet delivery was followed by a 10 sec timeout. Completing an FR on the incorrect nosepoke resulted in a 10 sec timeout with no pellet delivery. At the end of the 5-min S^D period, the rat was removed from the operant chamber and injected with either saline or drug and returned immediately to the chamber for the subsequent component. The number of components was randomized across days, ranging from 1 – 4 components. Multiple component training consisted of 1 – 4 injections of saline (saline, saline-saline, saline-saline-saline, or saline-saline-saline-saline) or 0-3 injections of saline followed by administration of training dose (drug, saline-drug, saline-saline-drug, saline-saline-saline-drug). Training dose was always administered in the last component.

Discrimination training continued until the following criteria were met: 1) >80% injection appropriate responding across components and 2) successful completion of first FR on injection-appropriate nosepoke. Testing sessions (as described below) were not initiated until training criteria were met for 5 consecutive days. Cocaine discrimination was achieved in X-Y sessions, depending on the rat. Fentanyl discrimination was achieved in X-Y sessions, depending on the rat.

Testing & Maintenance

Test sessions were conducted no more than 2 times per week; however, if training criteria were not met on any maintenance day, then three subsequent maintenance days were required in which the aforementioned criteria were met prior to re-initiating testing. Test sessions consisted of four components unless an animal failed to complete a single FR in components 1-3. Drugs were administered immediately prior to the start of each component as described above, and dose response curves were constructed by administering cumulative doses of drug at the start of each component. Full or complete generalization to a discriminative cue was defined as >85% of responding on the drug-associated nose poke and completing at least one FR.

Experimental Design.

In all rats, operant and discrimination (fentanyl vs saline *or* cocaine vs saline) training and dose effect curves for training drug and substitutions were evaluated repeatedly prior to surgery. Once initial dose effect curves were determined, SNI or sham surgery was performed. Rats were given 72 hours post-operative recovery as described above. Then, dose effect curves for training drug and substitutions were repeatedly evaluated over 4 mo.

Drugs

Cocaine and ketamine were obtained from the Univ Michigan Medical Hospital Pharmacy. Fentanyl, nalbuphine, and gabapentin were purchased from Sigma Aldrich (St. Louis, MO). Morphine was purchased as a 50 mg/mL solution from Henry Schein. SNC80 was generously gifted by Dr. Kenner C. Rice. Naltrexone, and quinpirole were obtained from Tocris Biosciences (Bristol, UK). Xylazine was obtained from Heartland Vet Supply (Hastings, NE). Carprofen (Rimadyl) was purchased from Zoetis (Parsippany, NJ).

Fentanyl citrate, morphine sulphate, cocaine hydrochloride, nalbuphine hydrochloride, gabapentin, naltrexone hydrochloride, quinpirole hydrochloride, and amphetamine sulfate were dissolved in sterile, physiological saline. Carprofen and xylazine were diluted in sterile water. SNC80 was dissolved in 3-5% 1 M HCl. SNC80 vehicle was 3% 1M HCl, and vehicle for all other drugs tested in discrimination procedures was saline.

Amphetamine, cocaine, gabapentin, ketamine, nalbuphine, quinpirole, and xylazine were given intraperitonially (i.p.) and all other drugs were delivered subcutaneously (s.c.).

Statistical Analysis & Data Presentation

Dose effect curves are presented as an average for each testing period: pre-surgery, 1-2, and 3-4 months post-surgery. Statistics were conducted in SPSS version 29. Data were analyzed using 4-way repeated measure ANOVAs with the following variables: sex (male, female); surgical status (sham, SNI); dose (varied across drugs but vehicle, 3-4 doses); and time (pre surgery, 1-2 or 3-4 months after surgery). Necessary corrections were made according to established Mauchly's W criteria. Analyses were conducted on % training drug responding as well as rates of responding for each drug, over time in cocaine or fentanyl trained groups. Main effects and interaction terms

necessary for interpretation are reported. To further examine main effect of time or dose, post hoc one-way ANOVAs with post hoc analyses were conducted. In these analyses, data were collapsed across non-significant main effects when possible (e.g., non-significant main effect of sex, data collapsed across sex).

4.3 Results.

4.3.1 Figure 4-1. % Fentanyl Responding

As shown in Figure 4-1, fentanyl produced dose-dependent interoceptive effects in both males (4-1A, D) and females (4-1F, J) prior to and after surgery, supported by a main effect of dose [$F(3, 60)=39.81$, $p<0.001$, $n2p=0.67$]. There was no main effect of time [$F(2, 40)=27.32$, $p=0.1$, $n2p=0.39$], sex [$F(1, 20)=0.01$, $p=0.92$, $n2p=0.001$], or surgical status [$F(1, 20)=2.69$, $p=0.12$, $n2p=0.09$]. However, there was a three-way interaction between time, dose, and surgical status [$F(6, 120)=2.93$, $p=0.01$, $n2p=0.13$]. This is likely explained by the small rightward shifts in the dose effect curves following surgery, and these are slightly greater in injured rats (Fig. 4-1D, J) than sham rats (Fig. 4-1A, F), particularly in male rats (Fig 4-1A, D). All other interaction terms failed to reach significance (p's>0.07). Collectively, these data suggest small, rightward shifts in the fentanyl dose effect curves in both sham and SNI rats.

As shown in Figure 4-1, morphine dose-dependently substituted for fentanyl in both males (Fig. 4-1B, E) and females (Fig. 4-1H, K) prior to and after surgery, supported by a main effect of dose [$F(2.16, 38.82)=345.64$, $p<0.001$, $n2p=0.95$]. There was no main effect of time [$F(2, 36)=0.17$, $p=0.84$, $n2p=0.009$],surgical status [$F(1, 18)=0.39$, $p=0.54$, $n2p=0.02$], or sex [$F(1, 18)=2.93$, $p=0.1$, $n2p=0.14$]. Though, there was a two-way interaction between dose and surgical status [$F(2.16, 38.82)=3.38$, $p=0.04$, $n2p=0.16$] such that there were small, rightward shifts in the

dose effect curves in injured animals compared sham rats. All other interaction terms failed to reach significance (p's>0.07). Collectively, these results suggest that there were small rightward shifts in the morphine dose effect curves in male injured animals.

As shown in Figure 4-1, nalbuphine dose dependently substituted for fentanyl in both male (Fig. 4-1C, F) and female (Fig. 4-1G, J) rats prior to and after surgery [F(2.45, 46.52)=27.02, p<0.001, n2p=0.59]. There was no main effect of sex [F(1, 19)=2.73, p=0.12, n2p=0.13], surgical status [F(1, 19)=0.89, p=0.36, n2p=0.045], or time [F(2, 38)=1.38, p=0.27, n2p=0.07]. However, there was a three-way interaction between dose, time, and sex [F(8, 152)=1.78, p=0.08, n2p=0.09], which is likely explained by the observed increases in sensitivity (i.e., leftward shift in the dose effect curve) to the discriminative stimulus effects of nalbuphine . Though, there was no dose by time by surgical status three-way interaction, suggesting the increased sensitivity to nalbuphine substitution is unrelated to SNI-induced hypersensitivity. The smallest increase in sensitivity was observed in the male sham group, but this effect was not significant [F(8, 152)=0.25, p=0.98, n2p=0.013]. All other interaction terms failed to reach significance (p's> 0.12). Collectively, these results suggest a change in sensitivity to nalbuphine over time independent of nerve injury status.

4.3.2 Figure 4-2. Rates of Responding in Fentanyl-Trained Animals

As shown in Figure 4-2, fentanyl dose dependently decreased rates of responding prior to and after surgery in both males (Fig. 4-2A, D) and females (Fig. 4-2G, J), reflected by a main effect of dose [F(2.45, 47.00)=56.83, p<0.001, n2p=0.75]. There was no main effect of time [F(1.49, 28.25)=0.03, p=0.93, n2p=0.002], surgical status [F(1, 19)=1.83, p=0.19, n2p=0.09], or sex [F(1 19) = 0.001, p=0.97, n2p=0]. However, there was a significant dose by sex interaction [F(2.47, 47.00)=5.87, p=0.003, n2p=0.24], suggesting the rate decreasing effects are slightly more

potent in female rats. There was no three-way interaction between sex, dose, and surgical status [F(2.47, 47.00)=0.57, p=0.61, n2p=0.03], suggesting that SNI-induced hypersensitivity is not responsible for this small sex difference. All other interaction terms failed to reach significance (p's>0.16). These data collectively suggest SNI-induced hypersensitivity did not induce a change in fentanyl-induced rate suppressant effects.

As shown in Figure 4-2, morphine dose dependently decreased rates of responding prior to and after surgery in both males (Fig. 4-2B, E) and females (Fig. 4-2H, K), reflected by a main effect of dose [f(2.29, 45.86)=51.88, p<0.001, n2p=0.72]. There was no main effect of time [F(2, 40)=0.70, p=0.50, n2p=0.03], sex [F(1, 20)=0.33, p=0.57, n2p=0.02], or surgical status [F(1, 20+=0.03, p=0.87, n2p=0.001], suggesting morphine induced rate suppressant effects did not largely differ across conditions and that SNI-induced hypersensitivity did not alter morphine-induced rate suppressant effects. All interaction terms failed to reach significance (p's>0.11).

As shown in figure 4-2, nalbuphine dose-dependently decreased rates of responding prior to and after surgery in both males (Fig. 4-2C, F) and females (Fig. 4-2I, L), reflected by a main effect of dose F(2.31, 43.93)=27.05, p<0.001, n2p=0.59]. There was no main effect of time [F(2, 38)=0.82, p=0.45, n2p=0.04], sex [F(1, 19)=2.41, p=0.14, n2p=0.11], or surgical status [F(1, 19)=1.02, p=0.32, n2p=0.05]. However, there was a significant interaction between time and dose, suggesting that some doses of nalbuphine were less rate suppressant over time (Fig. 2C, F, I, L). There was no three-way interaction between time, dose, and surgical status [F(4.33, 82.35)=0.39, p=0.82, n2p=0.02], indicating that the small shifts in nalbuphine dose response curves over time were not related to nerve injury. All other interaction terms failed to reach significance (p's>0.19).

4.3.3 Figure 4-3. Cocaine-induced interoceptive effects in presence or absence of chronic neuropathic pain

As shown in Figure 4-3, cocaine produced dose-dependent interoceptive effects in both males (Fig. 4-3A, C) and females (Fig. 4-3G, K), reflected by a main effect of dose [$F(4, 68)=120.59$, $p<0.001$, $n2p=0.88$]. There was no main effect of time [$F(2, 34)=1.08$, $p=0.35$, $n2p=0.06$], sex [$F(1, 22)=0.54$, $p=0.86$, $n2p=0.002$], or surgical status [$F(1, 22)= 1.53$, $p=0.48$, $n2p=0.03$], suggesting cocaine-induced interoceptive effects were not altered over time, by sex, or by SNI-induced hypersensitivity. However, there is a three-way time by dose by sex interaction [$F(4.00, 68.06)=0.89$, $p=0.06$, $n2p=0.13$], suggesting that cocaine was more potent in male than female rats prior to surgery. All other interaction terms failed to reach significance p's>0.13.

As shown in Figure 4-3, amphetamine dose-dependently substituted for cocaine in both males (Fig. 4-3B, E) and females (Fig. 4-3H, K), reflected by a main effect of dose [$F(2.20, 26.38)=17.80$, $p<0.001$, $n2p=0.6$]. There was no main effect of time [$F(2, 24)=1.76$, p0.19, $n2p=0.13$], sex [$F(1, 22) = 1.76$, $p=0.21$, $n2p=0.13$], or surgical status [$F(1, 22)=0.26$, $p=0.62$, $n2p=0.02$], suggesting that discriminative stimulus effects of amphetamine was not altered over time, by sex, or by SNI-induced hypersensitivity. Though, there was a two-way dose by time interaction, likely reflecting the small rightward shift in the amphetamine dose effect curves at some time point over the study. There was also a significant two-way dose by sex interaction [$F(2.20, 26.38)=3.79$, $p=0.03$, $n2p=0.24$], likely reflecting the slightly higher potency in female rats. All other interaction terms failed to reach significance p's>0.13.

As shown in Figure 4-3, quinpirole substitution produced low levels of cocaine appropriate responding prior to surgery in both males (Fig. 4-3C, F) and females (Fig. 4-3I, L). After surgery, quinpirole substitution produced higher levels of cocaine appropriate responding in both sham and

SNI groups, though to a greater extent in SNI groups. Quinpirole substitution was dose dependent, reflected by a main effect of dose [F(2.17, 22.86)=8.71, p= 0.001, n2p=0.44]. There was no main effect of sex [F(1, 11) = 0.18 p0.68, n2p=0.016] or surgical status [F(1, 11)=0.995, p=0.34, n2p=0.08]. There was no main effect of time [F(1.51, 16.63)=1.08, 0.34, n2p=0.09], though there was a significant interaction between dose and time [F(6, 66)=15.23, p<0.001, n2p=0.58], l and significant three-way interaction between dose, time, and surgery status [F(6, 66)=3.40, p=0.006, n2p=0.24], reflecting greater increases in quinpirole substitution for cocaine over time in the injured animals than sham (male Fig. 4-3C, F; female Fig. 4-3I, L).

4.3.4 Figure 4-4. Rates of responding in cocaine-trained animals

As shown in Figure 4-4, cocaine dose-dependently decreased rates of responding prior to and after surgery in both male (Fig. 4-4A, D) and female rats (Fig. 4-4G, K), reflected by a main effect of dose [F(2.70, 54.06)=77.81, p<0.001, n2p=0.80]. There was no main effect of time [F(2, 40)=1.63, p=0.21, n2p=0.8] or surgical status [F(1, 20)=0.73, p=0.40, n2p=0.04]. Cocaine more potently suppressed rates of responding in female rats than male rats, reflected by a main effect of sex [F(1, 20)=8.56, p=0.008, n2p=0.3]. All interaction terms failed to reach significance (p's>0.18). These data collectively indicate that SNI-induced hypersensitivity did not alter cocaine-induced decreases in rates of responding.

As shown in Figure 4-4, amphetamine dose-dependently decreased rates of responding prior to and after surgery in both male (Fig. 4-4B, D) and female rat (Fig. 4-4H, K), reflected by a main effect of dose [F(2.28, 45.51)=71.92, p<0.001, 0.78]. There was no main effect of time [F(2, 40)=1.62, p=0.21, n2p=0.08], surgical status [F(1, 20)=0.02, p=0.88, n2p=0.001], or sex [F(1, 20)=23.70, p=0.054, n2p=0.50]. The main effect of sex was close to significant, and there was a

two-way interaction between dose and sex [F(2.28, 45.51)=6.91, p=0.002, n2p=0.26], such that amphetamine was more rate suppressant in females (Fig. 4-4H, K) than males (Fig. 4-4B, E). All other interaction terms failed to reach significance (p's>0.27). Collectively these data suggest chronic neuropathic pain did not alter amphetamine-induced rate suppressant effects.

As shown in Figure 4-4, quinpirole dose-dependently decreased rates of responding prior to and after surgery in both male (Fig. 4-4C, F) and female rats (Fig. 4-4I, L), reflected by a main effect of dose [F(2.03, 40.61)=77.44, p<0.001, n2p=0.80]. There was no main effect of time [F(2, 40)=0.16, p=0.85, n2p=0.008] or surgical status [F(1, 20)=0.86, p=0.36, n2p=0.04]. There was a main effect of sex [F(1, 20)=24.21, p<0.001, 0.55] and a significant [two-way interaction between dose & sex: F(2.03, 40.61)=5.62, p=0.007, n2p=0.22], such that female rats (Fig. 4-4I, L) were more sensitive than males (Fig. 4-4C, F) to the rate decreasing effects of quinpirole]. All other interaction terms failed to reach significance (p's>0.21). Collectively these results suggest chronic neuropathic pain did not alter quinpirole-induced rate suppressant effects.

4.4 Discussion

The goal of the present study was to determine if chronic neuropathic pain altered the discriminative stimulus of opioid analgesics. Several previous studies in humans reported that acute, laboratory induced pain states decreased the magnitude of the pleasant subjective effects of opioids (Comer et al., 2010; Zacny & Bekman, 2004); however, we do not fully understand how chronic pain states may alter opioid-induced pain states. Discrimination training and pre-surgical testing of substitution dose-effect curves were completed prior to and after induction of chronic neuropathic pain or sham states over 4 months. We repeatedly evaluated dose effect curves to

determine if, within subject, chronic pain states alter the interoceptive effects of opioid analgesics over time.

First, as expected, fentanyl produced dose-dependent interoceptive effects in both male and female rats (Fig. 4-1) and dose-dependently decreased rates of responding (Fig. 4-2). Following sham or SNI surgeries, there were minor rightward shifts in the fentanyl interoceptive effects dose response curves, suggesting a small, but significant decrease in fentanyl-induced interoceptive effects independent of surgical status. There was no significant change in fentanyl induced rate suppressant effects in any groups over time (Fig. 4-2). Prior to and after surgery, morphine dose-dependently substituted for fentanyl in both males and females, as expected (Fig. 4-2). Similar to fentanyl, there were small but significant changes in effectiveness of individual doses of morphine 3-4 months after sham *or* SNI surgery in male rats, suggesting SNI-induced hypersensitivity failed to alter the substitution of morphine to the fentanyl discriminative stimulus. Collectively, these data suggest that SNI-induced hypersensitivity failed to alter the discriminative stimulus of fentanyl or morphine (high efficacy MOR agonists) in both male and female rats. These changes may are unlikely to be explained by previously reported pain-induced decreased expression and activity of MORs (Back et al., 2006; Campos-Jurado et al, 2019; Dong et al., 2019; Hou et al., 2017; Ji et al., 1995; Kaneuchi et al., 2019; Pol et al., 2006; Porecca et al., 1998; Thompson et al., 2018; Yamamoto et al., 2008; Zhang et al., 1998), as small shifts were observed in the sham groups as well (Fig. 4-1). These findings are inconsistent with previous human studies that have demonstrated that acute pain states decreased the magnitude of opioid analgesic-induced subjective effects (Comer et al., 2010; Zacny & Beckham, 2004); however, methodological differences such as type of pain (human-acute, escapable; present study- chronic, inescapable) may explain the observed differences in results.

Nalbuphine, a partial MOR agoinst, dose-dependently substituted for fentanyl prior to and after surgery in both males and females (Fig. 4-1) and dose-dependently decreased rates of responding (Fig. 4-2) as expected. After surgery, in both sham and SNI groups, nalbuphine was more potent, suggesting an increase in sensitivity to nalbuphine-induced interoceptive effects over time that is independent of SNI-induced hypersensitivity. Therefore, other factors may be responsible for the increased sensitivity to the discriminative stimulus of nalbuphine over time.

Rats began these studies in age-matched cohorts, but these studies were longitudinal and animals ended the experiments significantly older (~12 months) than they started (~2 months), so it is possible that increased age of subjects may contribute to the changes in sensitivity to the nalbuphine discriminative stimulus over time. Increased sensitivity to MOR agonist-induced subjective effects has been observed in older humans compared to younger humans (Cherrier et al., 2009; McLachlan et al., 2011; Scott et al., 1987), suggesting age increases sensitivity to opioid-analgesic induced subjective effects. The leftward shift in the nalbuphine dose effect curve is potentially related to increased age of animals over the course of discrimination experiments. Further, this increased sensitivity to the discriminative stimulus of nalbuphine is consistent with the observed increased sensitivity to the antihyperalgesic-like and antinociceptive-like effects of nalbuphine (see Ch. 2 Fig. 2-3) over time following both sham and SNI surgery. It is possible that we only observed an increased sensitivity in the discriminative stimulus of nalbuphine (but not morphine, fentanyl) as these assays may not be sensitive enough to detect small changes in higher efficacy MOR agonists. Future studies should carefully evaluate the reinforcing effects of partial MOR agonists over time in the presence or absence of pain states.

Collectively, SNI-induced hypersensitivity failed to alter the discriminative stimuli of MOR agonists (fentanyl, morphine, nalbuphine) (Fig. 4-1). While evaluating the impact of chronic

neuropathic pain states on the interoceptive effects of opioid analgesics was the primary goal of this study, we also examined the interoceptive effects of cocaine, an indirect dopaminergic agonist and a drug of abuse. In animals trained to discriminate 5.6 mg/kg cocaine from saline, cocaine and amphetamine produced dose-dependent increases in cocaine-appropriate responding prior to and after sham or SNI surgery (Fig. 4-3) and dose-dependently decreased rates of responding (Fig. 4-4). No effects of SNI-induced hypersensivity were observed on the interoceptive or rate decreasing effects of cocaine or amphetamine. Collectively, these results suggest that the interoceptive effects of indirect dopaminergic agonists were not altered by chronic neuropathic pain, suggesting no change in abuse potential. This is supported by the finding that chronic neuropathic pain did not alter the reinforcing effects of cocaine (Ch. 3, Figure 2-3, 2-4).

Prior to surgery, quinpirole produced low levels of cocaine-appropriate responding, consistent with previous studies (Katz & Witkin, 1982; Collins et al., 2014). Unexpectedly, after surgery, increased sensitivity to quinpirole was observed in all groups such that larger doses of quinpirole produced higher levels of cocaine-appropriate responding. While increased sensitivity was observed in sham *and* SNI groups, there was a greater increase in the SNI groups (Fig. 4-3); however, no changes in quinpirole induced rate decreasing effects were observed in any group (Fig. 4-4). The increased sensitivity to the quinpirole discriminative stimulus in chronic neuropathic pain was somewhat surprising. Increased quinpirole-induced cocaine-appropriate responding in both sham and SNI groups suggests that while SNI-induced hypersensitivity may contribute, other factors likely are involved. Future studies should directly evaluate the reinforcing effects of dopaminergic agonists in the presence or absence of chronic neuropathic pain.

Overall, the results presented in this study suggest that chronic neuropathic pain failed to alter the discriminative stimuli of MOR agonists, though changes in sensitivity were observed over

time (independent of surgical status). The present study also found that the discriminative stimulus of cocaine or amphetamine was not altered by presence of SNI-induced hypersensitivity, though quinpirole was more cocaine-like in both sham and SNI groups over time. Therefore, future experiments should directly evaluate 1) the abuse potential of multiple partial MOR agonists in the presence or absence of chronic neuropathic pain, as well as in aged rats and 2) directly evaluate the abuse potential of dopaminergic agonists in the presence or absence of chronic neuropathic pain.

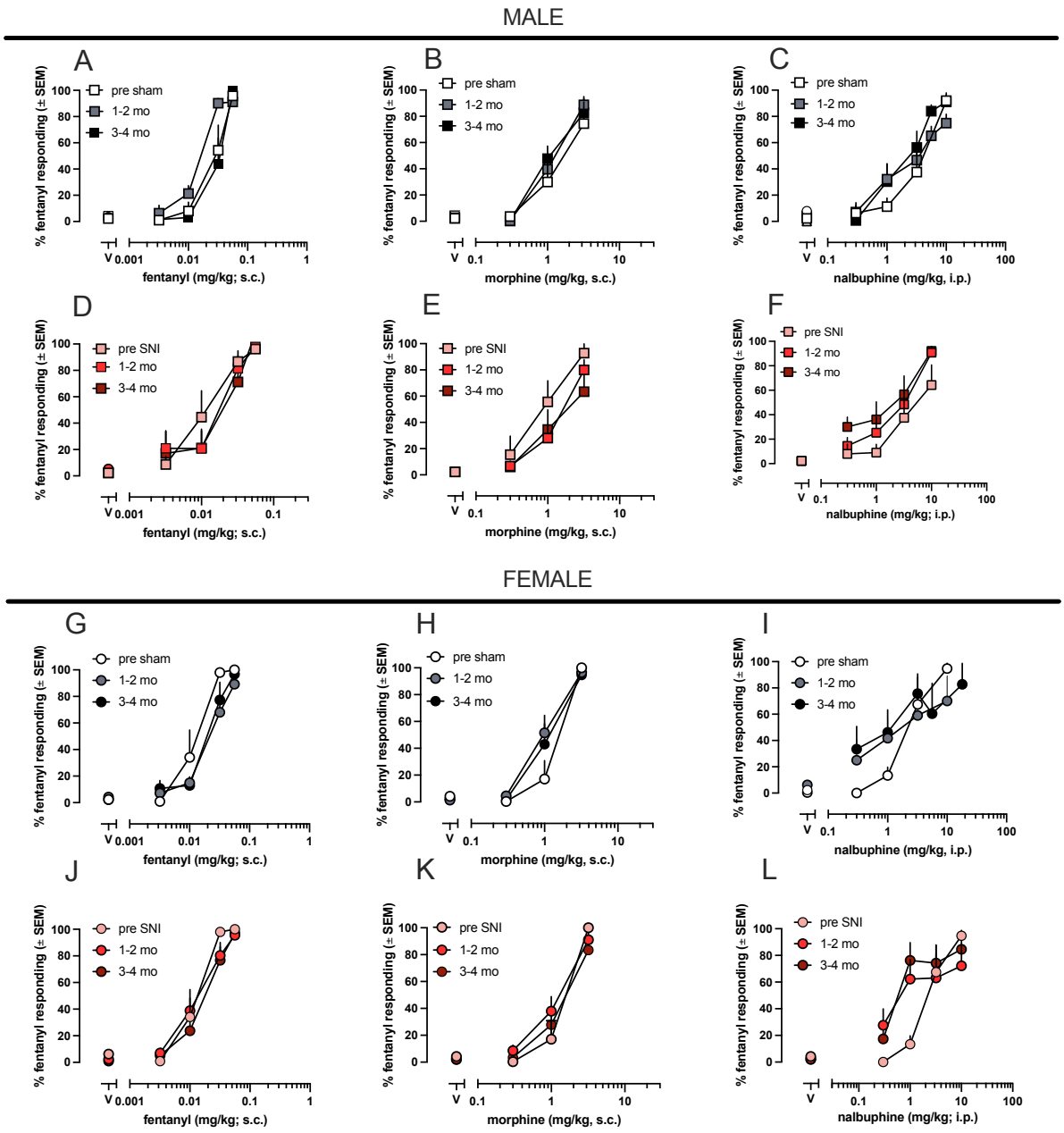

Figure 4-1. Interoceptive effects of opioid analgesics in males and females in the presence or absence of chronic neuropathic pain

In males, prior to either sham (A) or SNI (D) surgery fentanyl produced dose dependent increases in % fentanyl responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (A) or SNI surgery (D). Morphine produced dose dependent increases in %

fentanyl responding in male rats prior to sham (B) or SNI (E) surgery, and there were no significant shifts in the dose effect curves following sham (B) or SNI (E) surgery. Nalbuphine produced dose dependent increases in % fentanyl responding prior to sham (C) or SNI (F) surgery, and in both sham (C) or SNI (F) groups, leftward shifts were observed in the dose effect curves following surgery.

In females, prior to either sham (G) or SNI (J) surgery fentanyl produced dose dependent increases in % fentanyl responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (G) or SNI surgery (J). Morphine produced dose dependent increases in % fentanyl responding in male rats prior to sham (H) or SNI (K) surgery, and there were no significant shifts in the dose effect curves following sham (H) or SNI (K) surgery. Nalbuphine produced dose dependent increases in % fentanyl responding prior to sham (H) or SNI (L) surgery, and in both sham (H) or SNI (L) groups leftward shifts were observed in the dose effect curves following surgery.

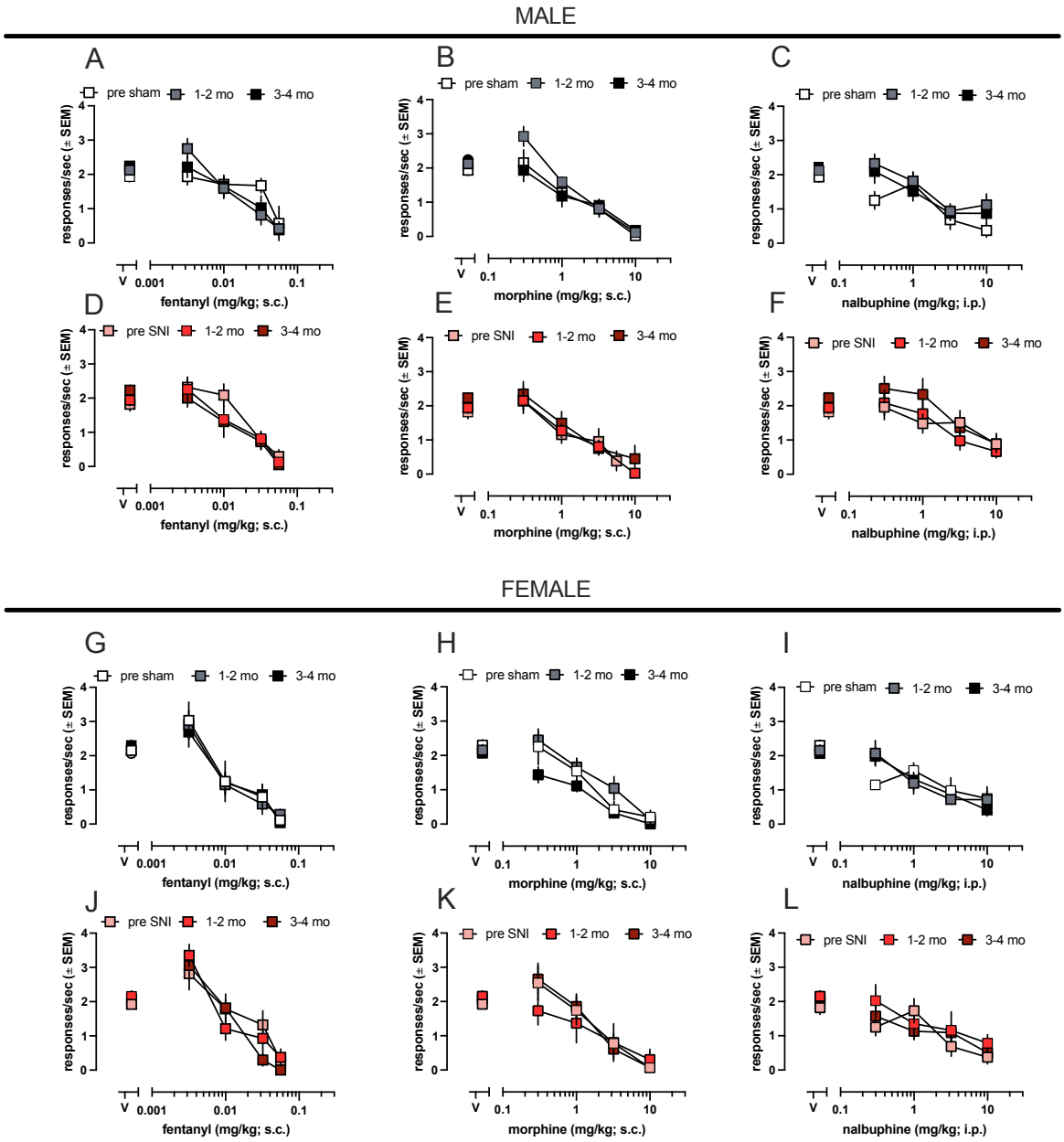

Figure 4-2. Rate suppressant effects of opioid analgesics in the presence or absence of chronic neuropathic pain

In males, prior to either sham (A) or SNI (D) surgery fentanyl produced dose dependent decreases in rates of responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (A) or SNI surgery (D). Morphine produced dose dependent decreases in rates of responding in male rats prior to sham (B) or SNI (E) surgery, and there were no significant shifts

in the dose effect curves following sham (B) or SNI (E) surgery. Nalbuphine produced dose dependent decreases in rates of responding prior to sham (C) or SNI (F) surgery, and in both sham (C) or SNI (F) groups, leftward shifts were observed in the dose effect curves following surgery.

In females, prior to either sham (G) or SNI (J) surgery fentanyl produced dose dependent decreases in rates of responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (G) or SNI surgery (J). Morphine produced dose dependent decreases in rates of responding in male rats prior to sham (H) or SNI (K) surgery, and there were no significant shifts in the dose effect curves following sham (H) or SNI (K) surgery. Nalbuphine produced dose dependent decreases in rates of responding prior to sham (H) or SNI (L) surgery, and in both sham (H) or SNI (L) groups leftward shifts were observed in the dose effect curves following surgery.

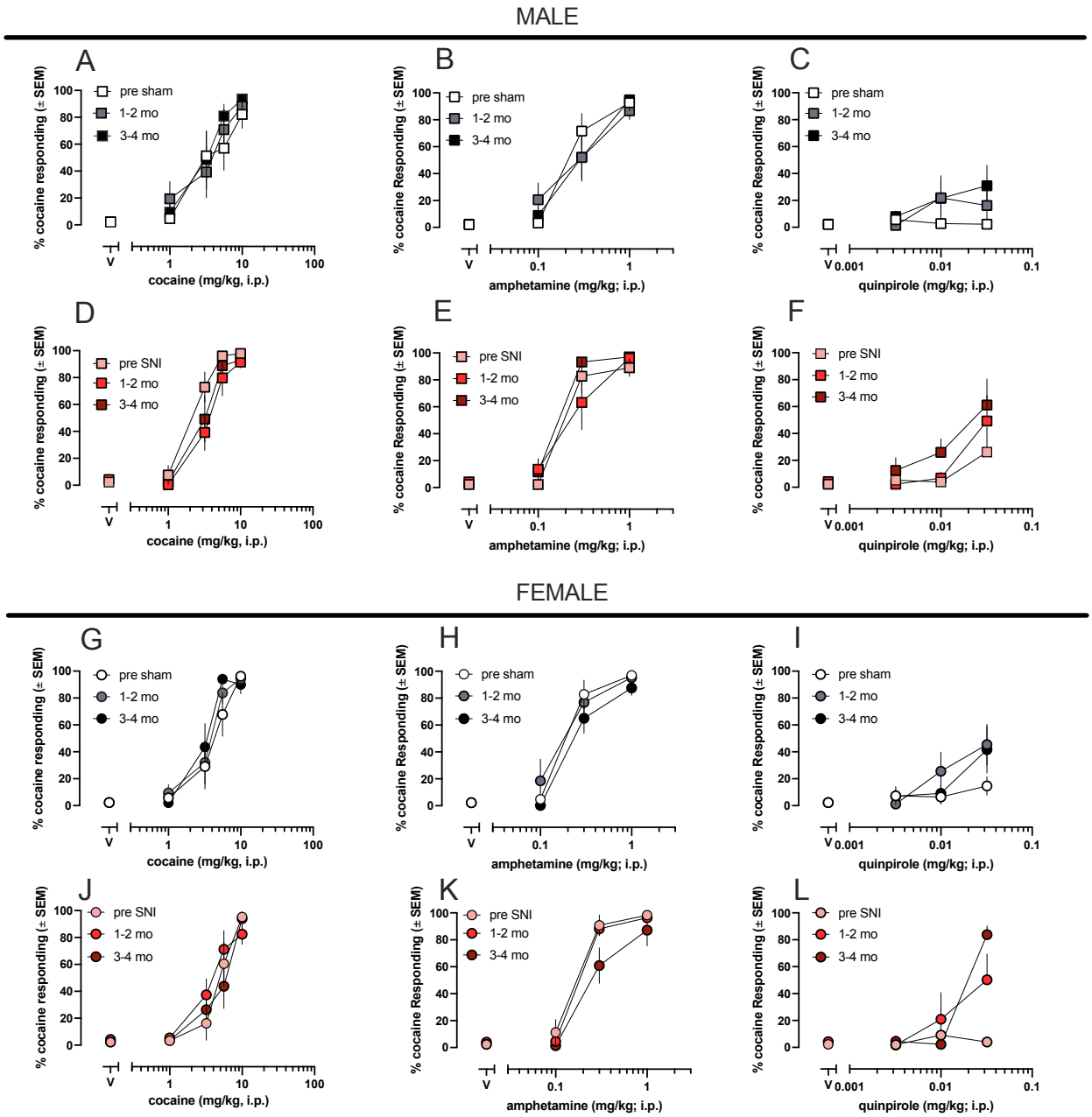

Figure 4-3. Interoceptive effects of dopaminergic drugs in the presence or absence of chronic neuropathic pain

In males, prior to either sham (A) or SNI (D) surgery cocaine produced dose dependent increases in % cocaine responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (A) or SNI surgery (D). Amphetamine produced dose dependent increases in %

cocaine responding in male rats prior to sham (B) or SNI (E) surgery, and there were no significant shifts in the dose effect curves following sham (B) or SNI (E) surgery. Prior to sham (C) or SNI (F) surgery, quinpirole produced low levels (<25%) of % cocaine responding. Following sham (C) or SNI (F) surgery, quinpirole produced significantly higher levels of % cocaine responding in sham (C) and SNI (F) groups, albeit lower levels in sham.

In females, prior to either sham (G) or SNI (J) surgery, cocaine produced dose dependent increases in % cocaine responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (G) or SNI surgery (J). Amphetamine produced dose dependent increases in % cocaine responding in female rats prior to sham (H) or SNI (K) surgery, and there were no significant shifts in the dose effect curves following sham (H) or SNI (K) surgery. Prior to sham (I) or SNI (L) surgery, quinpirole produced low levels (<25%) of % cocaine responding. Following sham (I) or SNI (L) surgery, quinpirole produced significantly higher levels of % cocaine responding in sham (I) and SNI (L) groups, albeit lower levels in sham.

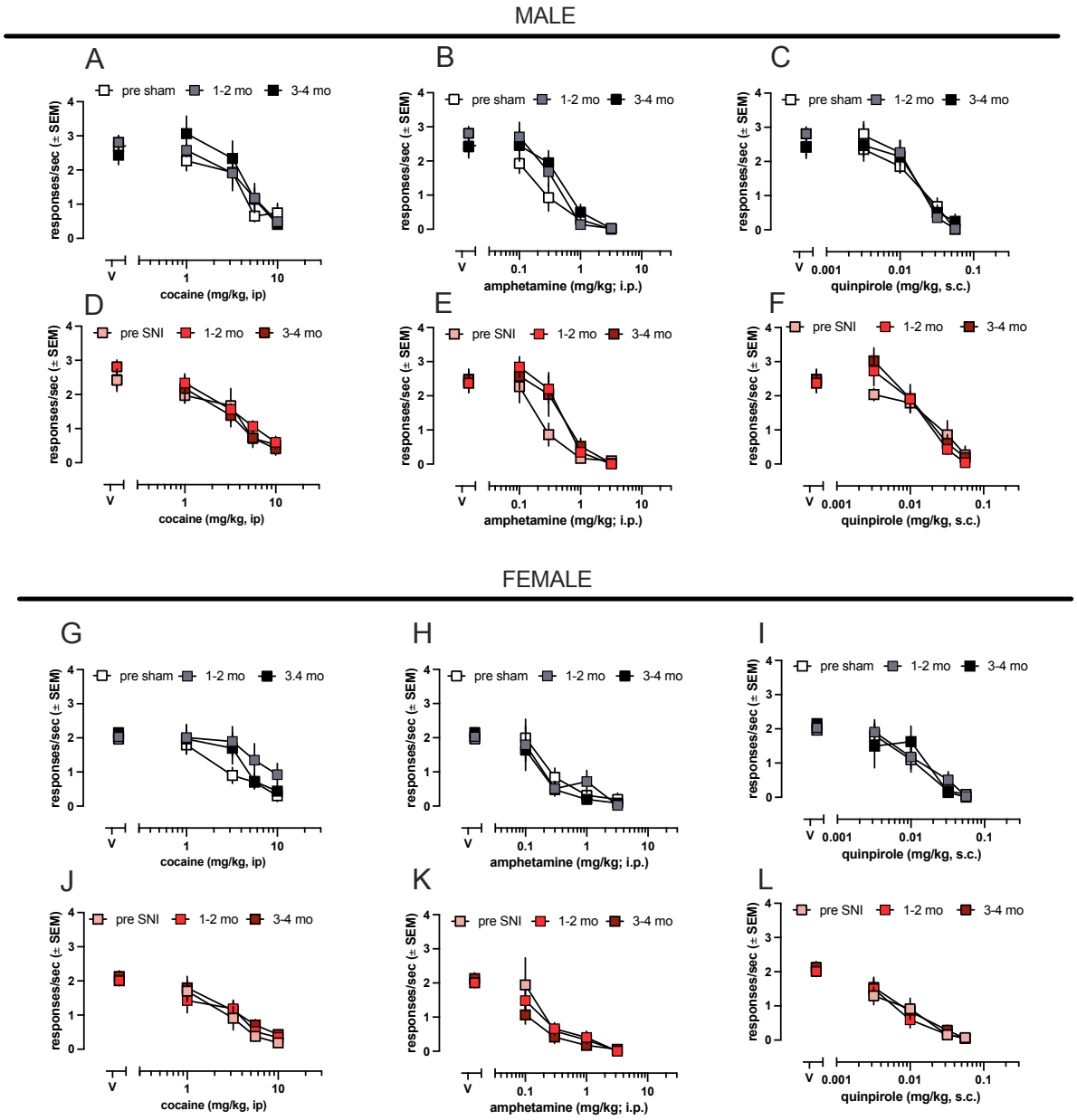

Figure 4-4. Rate suppressant effects of dopaminergic drugs in the presence or absence of chronic neuropathic pain

In males, prior to either sham (A) or SNI (D) surgery cocaine produced dose dependent decreases in rates of responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (A) or SNI surgery (D). Amphetamine produced dose dependent decreases in rates of responding in male rats prior to sham (B) or SNI (E) surgery, and there were no significant

shifts in the dose effect curves following sham (B) or SNI (E) surgery. Quinpirole produced dose dependent decreases in rates of responding prior to sham (C) or SNI (F) surgery, and in both sham (C) or SNI (F) groups, no significant shifts were observed in the dose effect curves following surgery.

In females, prior to either sham (G) or SNI (J) surgery cocaine produced dose dependent decreases in rates of responding, and there were no significant shifts in the dose effect curves after up to 4 months of sham (G) or SNI surgery (J). Amphetamine produced dose dependent decreases in rates of responding in male rats prior to sham (H) or SNI (K) surgery, and there were no significant shifts in the dose effect curves following sham (H) or SNI (K) surgery. Quinpirole produced dose dependent decreases in rates of responding prior to sham (H) or SNI (L) surgery, and in both sham (H) or SNI (L) groups no significant shifts were observed in the dose effect curves following surgery.

Chapter 5 General Discussion

5.1 Goal of experiments

The experiments described in this dissertation sought to determine if chronic neuropathic pain altered sensitivity to the behavioral effects of opioid analgesics with the ultimate goal of evaluating whether chronic pain increases susceptibility to OUD. In all experiments, opioid analgesic-induced behaviors were examined prior to the induction of pain, then animals underwent sham or spared nerve injury (SNI) surgery and the effects of opioid analgesics were evaluated post-operatively. This allowed us to assess potential changes in the antinociceptive- or antihyperalgesic-like effects, rate suppressant effects, reinforcing effects, and interoceptive effects of opioid analgesics over time in a within-subject study design.

5.2 Summary of Results

Overall, the experiments outlined in this dissertation demonstrated that SNI-induced hypersensitivity did not alter behavioral effects of high efficacy opioid analgesics, such as fentanyl and morphine. Small, but statistically significant, decreases in the potency of fentanyl and morphine over time were observed in all assays (antinociceptive-like effects, antihyperalgesic-like effects, reinforcing effects, interoceptive effects); however, these effects were observed in both sham and SNI groups. These data suggest that the small decreases in the sensitivity to high efficacy opioid analgesic induced behavioral effects over time were not induced by the presence of a chronic pain state.

Unlike that observed with fentanyl and morphine, there were robust changes in the sensitivity to the behavioral effects of a partial MOR agonist, nalbuphine. In summary, we observed increases in sensitivity to nalbuphine induced antinociceptive-, antihyperalgesic-like, and discriminative stimulus effects of nalbuphine. However, we observed a decrease in sensitivity to nalbuphine-induced rate suppressant effects, as demonstrated by a rightward shift in the nalbuphine dose effect curve as measured in schedule-controlled responding experiments (Chapter 2). Again, the changes in the effects of nalbuphine were observed in both sham and SNI groups in all assays, suggesting SNI-induced hypersensitivity does not explain changes in the nalbuphine dose effect curves over time.

Across all of these studies, chronic nerve injury or neuropathic pain had no effect on the behavioral effects of MOR agonists, such that there were no changes in the MOR agonist-induced effects within subject (pre- vs post-SNI) or between groups (sham vs SNI). In addition, chronic nerve injury also failed to alter cocaine-induced behaviors indicative of its abuse liability, such as interoceptive and reinforcing effects. Together, these findings demonstrate that chronic neuropathic pain itself does not alter the behavioral effects of MOR agonists and stimulants. Thus, if chronic pain is indeed a risk factor for developing OUD, then it is likely independent of neuropathic-induced changes in the pharmacological activity of MOR agonists. However, chronic pain as a risk factor for OUD could be due to other factors, such as exposure and access to MOR agonists, repeated use of MOR agonists, or changes pain-related mood states. Future studies should investigate these other possible pain-induced risk factors for OUD in separate studies.

However, there are caveats that may account for the lack of effect of chronic nerve injury on the MOR agonist-induced behaviors. For example, while SNI injury induces mechanical hypersensitivity, altered gait, and limb retraction while standing, rats may not actually be in pain.

Injured animals gain weight over time, groom, interact with enrichment, run, jump, and play similar to behaviors observed in sham rats. Additionally, nerve injury did not appear to alter responses to thermal noxious stimuli, since there were no differences in response latency in the hot plate assay (consistent with previous studies- Wen et al., 2007). It is possible that SNI surgery induces decreased limb function or other sensations, e.g., numbness, rather than chronic, ongoing pain. It is also possible that sham rats experience long-lasting post-operative pain from the muscle incision alone, which produced pain-related changes in the behavioral effects of MOR agonists. Therefore, future studies should consider using an additional control of naïve rats or no surgery group to evaluate changes in the behavioral effects of MOR agonists over time.

Another caveat is the type of pain utilized in the current studies. Perhaps chronic neuropathic pain does not produce long-lasting, consistent alterations in the MOR system or does not produce persistent changes in the MOR number and function. First, when previous studies have evaluated the effect of acute pain states on MOR expression or function, animals are sacrificed, and brains are collected while the pain state is ongoing. We have relatively little understanding about the time course of MOR expression following exposure to acute pain states. Future work should directly examine how long-lasting pain-induced decreases in MOR expression or function by evaluating MOR number and function at multiple time-points following induction of pain states.

While chronic nerve injury did not induce changes in the behavioral effects of MOR agonists, there were interesting and robust bidirectional changes in sensitivity to the behavioral effects of nalbuphine. In the schedule maintained responding assay (Chapter 2, Figure 7), nalbuphine dose-dependently decreased rates of responding in all groups prior to surgery. However, 1-3 months after surgery, there were rightward shifts in the dose effect curves in both

sham and SNI group, suggesting decreased sensitivity to rate-decreasing effects of nalbuphine. Approximately 4-6 months after surgery, we observed at least a 10 fold shift in the rate decreasing effects of nalbuphine in male rats; however, in female rats, doses up to 100 mg/kg nalbuphine failed to significantly decrease rates of responding. Larger doses were not tested in female rats due to solubility limitations. These results suggest a decrease in the potency, and possibly efficacy, of nalbuphine over time.

The decrease in sensitivity to fentanyl-, morphine-induced behavioral effects across all studies and the nalbuphine-induced rate suppressant effects is consistent with previously reported decreased MOR expression, measured by mRNA (Dong et al., 2019; Hou et al., 2017; Pol et al., 2006), protein levels (Back et al., 2006; Ji et al., 1995; Kaneuchi et al., 2019; Zhang et al., 1998), PET (Thompson et al., 2018), or [^{3}H] DAMGO binding in tissue homogenates (Ozaki et al., 2002; Porecca et al., 1998). Further, several studies have examined MOR activation via GTPγS assays (Obara et al., 2010; Ozaki et al., 2002) in pain states and shown decreased activation, but not all (Porecca et al., 1998). These studies have shown lower levels of MOR expression or activation in the spinal cord and brain regions soon after surgery (e.g., within 5 days of surgery) as well as up to 3 months after, suggesting the changes in MOR levels and function are persistent, though, only Thompson et al., 2018 measured more than 2 weeks into pain (3 months). Partial agonists, such as nalbuphine, should be more affected by loss of receptor number than full agonists. The observed small, but significant, decreases in sensitivity to fentanyl or morphine (higher efficacy agonists) over time are also consistent with decreases in MORs. However, decreased MOR expression or activation is not consistent with the *increased* sensitivity to the antihyperalgesic and discriminative stimulus effects of nalbuphine. All observed effects are also independent of pain as the changes in sensitivity to MOR agonists were observed in sham groups as well. There are a number of possible

explanations for the increased sensitivity to the behavioral effects of nalbuphine over the time course of this study.

First, MOR populations in different brain regions or circuits mediating these effects of nalbuphine could be differentially altered over time, either due to a function of rat age or repeated exposure to MOR agonists. The current experiments were longitudinal, so rats were approximately 10-13 months older at the experimental endpoint (e.g., 4-6 post-surgery determinations of drug effects), nearly 1/3 -1/2 of their life span. Previous studies have demonstrated age-related increased sensitivity to the effects of partial MOR agonists in both humans and rodents. In humans, studies have reported that opioid-induced analgesic effects are increased in elderly patients compared with younger patients (van Ojik et al., 2012), and one study reported increased MOR binding older humans (Zubieta et al., 1999). Similarly, there are also several studies suggesting that the subjective effects of opioid analgesics are enhanced in older people (50+ years old) than younger (~18-30 years old) people (Cherrier et al., 2009). In rats, one study sought to characterize the effects of age on opioid-analgesic induced antinociceptive effects as measured by the hot plate assay. Antinociceptive-like effects of morphine, levorphanol, butorphanol, nalbuphine, and nalorphine (listed most to least efficacious) were tested within-subject at approximately 2 and 24 months old. This study reported increased sensitivity at 24 months compared to 2 months to buprenorphine-, nalorphine-, butorphanol- and nalbuphine-induced antinociceptive effects while no significant change in morphine- or levorphanol-induced effects was observed (Smith & Grey, 2001). It is possible that the assays used are not sensitive enough to detect small changes in the antinociceptive-like effects of higher efficacy agonists. This study specifically demonstrates increased sensitivity to partial agonist-induced antinociceptive-effects in aged rats (Smith & Grey, 2001). The results from this study are consistent with the results discussed in this dissertation such

that increased sensitivity was observed for partial (low efficacy) MOR agonists, but not high efficacy MOR agonists. This study only included male rats, but we observed increased sensitivity to the partial agonist nalbuphine in both male and female rats. Drug discrimination assays are generally run for the duration of a rodent's lifespan, and previous studies suggest there is no robust change over time in the discriminative stimulus effects of MOR agonists (Colpaert et al., 1999 for review); however, few studies have evaluated the effects of partial MOR agonists over time.

Another possible explanation is that activity at other opioid receptor types (non-MOR) may enhance the antihyperalgesic and discriminative stimulus properties of nalbuphine. Nalbuphine is approximately 9-fold more selective for MORs than KORs (Santos et al., 2023) and activates KORs (~47%) to a greater extent than MORs (~18%). While KOR expression has been previously demonstrated to be increased in pain states (Liu et al., 2022 (CFA, PNI); Xu et al., 2004), previous work has demonstrated that the interoceptive effects of MOR and KOR agonists are distinct, such that there were low levels of cross-generalization (Negus, Picker, & Dykstra, 1990). Additionally, the discriminative stimulus effects of nalbuphine were shown to be mediated by MORs (Gereak and France, 1996). Therefore, it is unlikely that nalbuphine-induced KOR activation contributed to the increased sensitivity to the discriminative stimulus effects of nalbuphine in the current study. Alternatively, KOR agonists have been demonstrated to effectively attenuate neuropathic pain-like states in rodents (Hall et al., 2016; Paton et al., 2022). As the increased sensitivity to nalbuphine-induced antihyperalgesic effects were observed in both sham and SNI groups, it is unlikely that pain-induced KOR activation contributes to the increased sensitivity. Future experiments could directly test this with a pretreatment of a KOR selective antagonist.

Third, increased sensitivity to behavioral effects of nalbuphine could be some evidence of sensitization. Previous work has demonstrated that sensitization is usually not related to changes

in the drug target (e.g., MOR's), but rather is due to alterations in downstream signaling and effects across an entire neural circuit. Several studies have suggested that repeated administration of drug and some period of abstinence produce sensitization to locomotor stimulating properties of both opioid analgesics and stimulants (for review see: Delage et al, 2023). Further, previous studies have generally used daily injections of opioids, followed by abstinence to study behavioral sensitization. In the evaluation of opioid analgesic induced analgesic-like effects, animals were intermittently exposed to opioids. One dose response curve was performed each week, and 2-4 weeks out of each month involved opioid analgesic administration. While these experiments did not use the exact same dosing schedule, there was intermittent exposure over time. Therefore, nalbuphine may have become more potent over time in some assays, but not others, due to sensitization-related mechanisms.

Finally, it is also possible that, as nalbuphine became less rate suppressant overtime, then antihyperalgesic and discriminative stimulus effects of nalbuphine were unmasked. In the case of drug discrimination assays, decreased behavioral disruption may have revealed an increase in the discriminative stimulus of nalbuphine (Withey et al., 2020). We used a pain-elicited behavioral assay to measure antihyperalgesic- and antinociceptive-like effects of opioid analgesics, and these assays can be sensitive to behavioral disruption. Perhaps, we observed a very slow onset of behavioral sensitization with this dosing schedule.

Previous research has demonstrated sex differences in many opioid-induced behaviors including potency of antinociceptive or antihyperalgesic effects (Barrett, Smith, & Picker, 2002; Cook et al., 2000; Cook & Nickerson, 2005; Craft et al., 2001; Peckham & Traynor 2005, 2006; Terner et al., 2002; 2005; for review- Craft et al., 2003) and addiction related behaviors such as acquisition of self-administration, escalation of intake, and motivation to seek drug (for review:

Becker & Koob, 2016). Previous studies have examined opioid-induced analgesic effects in the SNI model, but these studies have only used male rodents (Erichsen & Blackburn-Munro, 2008; Zhao et al., 2004). Similarly, most previous studies testing the impact of pain states on reinforcing effects of opioid analgesics have been completed in male rats only (Martin et al., 2007; Lyness et al., 1989; Woller et al., 2014; Wade et al., 2003; Kupers & Gybel, 1995; Hipólito, et al., 2015; Hou et al., 2015; & Colpaert et al., 2001). Therefore, in the present study, we evaluated males and females in all experiments in order to fill this gap in the literature and have a broader understanding of the interaction between chronic pain and opioid-analgesic induced behavioral effects. Unfortunately, we were unable to directly evaluate sex differences in the self-administration studies due to catheter failures in female rats. Across the studies presented in this dissertation, we observed very few sex differences. We observed a small increase in potency of morphine in females compared to males in both Randall Selitto and hot plate assays. Nalbuphine was slightly more effective in female sham rats compared to male sham rats, with no difference in SNI groups. No sex differences were observed in fentanyl-induced effects. While the current project focused on sex differences in the behavioral effects of MOR agonists, we also observed sex differences in the behavioral effects of other drugs. For example, THC- and cocaine-induced antihyperalgesic effects were more potent in female as compared with male rats, whereas the KOR agonist EKC was more potent in male than female rats. In rats trained to discriminate cocaine injections, amphetamine and quinpirole were more potent at rate suppressant effects in females than males. Interestingly, up to doses of 180 mg/kg, gabapentin failed to decrease rates of responding in male, but not female, rats. Several experimental details may contribute to the lack of sex differences in the present studies.

First, there is a decreased receptor reserve for female rodents compared to male rodents (Peckham & Traynor 2005, 2006), so sex differences may be most easily detected in pain assays involving a more intense noxious stimulus (e.g., hot plate) than a less noxious stimulus (e.g., mechanical hypersensitivity) or effects of lower efficacy agonists. One study directly examined these variables and observed the most robust sex differences with partial agonists or a more intense noxious stimulus (55 ℃ vs 50) (Cook et al., 2000). Perhaps we did not observe robust sex differences in the evaluation of mechanical hypersensitivity as this is a lower efficacy requiring assay; however, this does not explain the lack of sex differences observed in this study in the hot plate assay. While pain intensity and opioid efficacy contribute to sex differences in opioid potency, these factors do not explain all differences.

The type of pain state used may also be important. Many of the previous studies demonstrating sex differences have used nociceptive or inflammatory pain states, and relatively few studies have systematically tested dose effect curves of opioid analgesics in male and female rats experiencing neuropathic pain. More specifically, one previous study using the SNI model has demonstrated female rats had lower paw withdrawal thresholds than male rats; however, opioids were not tested (Alhstrom et al., 2021). Studies testing opioid analgesic-induced effects have used male rats only (Erichsen & Blackburn-Munro, 2008; Zhao et al., 2004). Collectively, little is known about opioid-agonist induced antinociceptive- or antihyperalgesic-like effects in chronic neuropathic pain states.

5.3 Overall conclusions

Collectively, over time, there were minimal rightward shifts in the potency of higher efficacy opioid analgesics (fentanyl, morphine). Overall, these changes were observed in the sham

and SNI groups, suggesting chronic neuropathic pain is not responsible for the observed shifts in sensitivity. This suggests that chronic neuropathic pain did not alter the abuse potential of high efficacy opioid analgesics; however, caution should still be used in prescribing opioids for chronic pain.

More robust changes in nalbuphine-induced behavior were observed over time. It is possible that these observed changes in nalbuphine (increased sensitivity to analgesic-like effects, interoceptive effects) could suggest increased abuse potential of *partial agonists* over time, or in pain states. Future studies should test the influence of pain states and age on the abuse potential of partial agonists. If future studies demonstrated no change in abuse potential or other adverse effects of partial agonists and verified increased sensitivity to antinociceptive- or antihyperalgesic-like effects over time, this may suggest partial agonists would be beneficial in the treatment of chronic neuropathic pain.

While the present studies found no SNI-induced changes in opioid-induced effects, chronic pain should increase patient's (legal) *access* to opioids as opioids are mainly prescribed for the treatment of pain. Studies have shown that increased access to the reinforcer (e.g., lower effort requirements), or decreased access to alternate reinforcers (e.g., higher effort requirements can shift choice) (Bickel et al., 2014). In cases of clinically significant pain, pain itself often decreases the frequency of behaviors such as walking and movement as well as ability to work, and this is referred to as pain depressed behavior. Under these conditions, it is likely that the patient may less frequently engage with other social reinforcers (e.g., meeting a friend, going to work) (Martin et al., 2004; Morgan et al., 2008).

One of the 11 DSM-5 criteria for OUD diagnosis is "important social, occupational, or recreational actives are given up or reduced because of opioid use" (Boyd et al., 2020). This

criterion could be confounded by pain itself decreasing engagement with other social reinforcers. Therefore, while the present studies demonstrated that chronic neuropathic pain did not alter the *abuse potential* of opioids, it is possible that other pain-related changes in behavior may increase likelihood of opioid use or accessibility of the opioid reinforcer, and therefore risk of developing OUD (for more detailed review, see Nazarian, Martin, & Negus, 2021).

Future Directions

Many future studies should be completed to have a better, more broad understanding of the complex relationship between abuse potential and pain states. Future studies should directly determine 1) if these reported changes in sensitivity are specific to nalbuphine-induced behavioral effects or if increased attenuation of mechanical hypersensitivity is observed over time with other partial agonists. Buprenorphine would be an excellent comparison as buprenorphine is FDA approved for the treatment of pain and OUD. Further, buprenorphine is a partial MOR agonist, like nalbuphine; however, buprenorphine is a KOR antagonist (Volpe et al., 2001; Huang et al., 2011). Evaluation of buprenorphine-induced behavioral effects could shed light on possible contributions of KOR agonist activity in the behavioral effects of nalbuphine. 2) The abuse potential of a partial MOR agonist needs to be directly evaluated in the presence or absence of chronic neuropathic pain as well as over time. Further, behaviors related to other stages of the addition cycle should be evaluated. Small, but significant, shifts were observed in the self-administration of fentanyl (independent of nerve injury), and it is possible that larger changes would be observed in the abuse potential of a partial MOR agonist. Further, we examined interoceptive and reinforcing effects, though it would be useful to know if chronic pain increases the reinforcing properties of opioid-paired cues, motivation to seek opioid (progressive ratio), or

physical dependence and withdrawal symptoms. Previous work suggests acute, short acting pain states did not alter opioid-food choice; however, future studies could examine if chronic pain states alter opioid choice or examine if chronic pain would alter opioid-social interaction choice. 3) The effect of age on opioid analgesic-induced behavioral effects should be systematically studied in surgery-naive rats. Similar longitudinal designs could be used to evaluate age as a within-subject variable. 4) Experiments should directly evaluate the extent of tolerance development following repeated administration of MOR agonists in order to determine if tolerance to MOR agonist induced effects is observed to the same extent in a) older and younger animals and b) SNI and sham groups. Next, we did not observe changes in sensitivity to cocaine in the presence of SNI-induced hypersensitivity but quinpirole produced higher levels of cocaine-appropriate responding over time in SNI groups. Therefore, 5) the abuse potential of dopaminergic agonists should be evaluated in the presence or absence of SNI-induced hypersensitivity. Finally, 6) a more complete understanding of SNI-induced neurobiological changes would be helpful. Previous studies have examined if SNI-induced hypersensitivity altered expression of MORs or endogenous opioid peptides in various brain regions; however, it would be useful to have data from later timepoints in pain (latest 3 months; Thomspon et al., 2018) as well as an idea if total receptor number is changed. I collected the brains from my drug discrimination and schedule maintained responding animals when experiments ended, and they are in storage. These brains were collected 4-6+ months after surgery, and they should be examined in future studies.

Overall, the data presented in this dissertation offer evidence that chronic neuropathic pain did not significantly alter the abuse potential of opioid analgesics. The data presented in this dissertation also suggest that evaluation of multiple behavioral effects over time is useful. No single pre-clinical assay can capture the complexities of how drugs alter behavior in humans;

however, simultaneous evaluation of independent, related behaviors offers a more complete understanding.

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