

REWARD-RELATED BEHAVIORAL EFFECTS OF PRESCRIPTION
OPIOIDS AS A FUNCTION OF PUTATIVE ACUTE AND
CHRONIC PAIN-LIKE STATES IN MALE
AND FEMALE C57BL/6 MICE

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ABSTRACT

Pain is a leading cause of disability and the most common reason for clinical care. The field of pain research has focused on sex differences in recent years with an expansive body of literature demonstrating sex-related differences in pain behavior and responsiveness to pharmacological interventions. Prescription opioids are potent analgesics and the mainstay for the clinical management of moderate-to-severe acute and chronic pain conditions. However, long-term clinical use of prescription opioids for chronic pain remains controversial due to concerns about severe adverse effects, including tolerance, dependence, and addiction associated with opioid use. The non-medical use and abuse of prescription opioids has become a public health crisis, the problem even arising in a subset of chronic pain patients receiving opioid therapy. The vulnerability factors, specifically the role of pain in the propensity to abuse prescription opioids, are poorly understood. The present research project sought to investigate the reward-related behavioral effects of prescription opioids as a function of pain in male and female mice by incorporating acute (acetic acid-induced) visceral nociceptive and chronic chemotherapy (paclitaxel)-induced peripheral neuropathic pain models. Sexually dimorphic variations in the sensitivities of mice to nociceptive and allodynic behaviors were initially assessed using the two pain models. Following that, two prescription opioids, morphine and oxycodone were examined under both pain contexts and the capacity of the two prescription opioids to produce reward-related behavioral effects were measured using drug discrimination, conditioned place preference, and intravenous drug self-administration procedures. The presence of acute pain but not chronic pain

selectively attenuated the discriminative stimulus effects of morphine in male mice. The magnitude of modulation of the stimulus effects of opioids by acute pain were further observed to be inversely related to the relative intrinsic antinociceptive effectiveness of the two opioids in reversing the acute pain (morphine < oxycodone), and sex-specific sensitivities of mice to opioid-induced antinociception (M < F). In contrast, while no change was observed in opioid-reward as a function of the acute pain in both sexes, the presence of paclitaxel-induced chronic pain opioid-selectively and dose-selectively enhanced the conditioned rewarding effect of morphine (0.3 mg/kg dose), and the effect was more pronounced in male relative to female mice. These data were further supported by the self-administration results, in that the reinforcing efficacy (breakpoints under progressive ratio (PR) responding) and the incentive-motivational salience of morphine significantly increased in the presence of chronic pain in male mice, relative to the saline-treated control male mice. However, female mice revealed increased sensitivity to morphine-induced reinforcing effects regardless of the presence or absence of chronic pain. Overall, the converging empirical evidence presented here suggest that these models provide preclinical tools to further understand the overlapping neurobiology of pain and opioid abuse, the behavioral effects of prescription opioids, and advance the development of novel sex-specific pain therapeutics with low addiction liability.

DEDICATION

I dedicate this research to my Grandfather, Ramani Thatha (deceased) and my friend at graduate school, Gladys Corley (deceased) in honor of having battled cancer (“pain”) with courage and inspired many hearts in so many ways.

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LIST OF ABBREVIATIONS

CPP – Conditioned Place Preference
FR – Fixed Ratio
PR – Progressive Ratio
VTA – Ventral Tegmental Area
PAG – Periaqueductal Gray
PBn – Parabrachial Nucleus
SNP – Single Nucleotide Polymorphism

CHAPTER 1

INTRODUCTION

Acute and Chronic Pain

Epidemiology and Treatment with Prescription Opioids

According to the International Association for the Study of Pain (IASP), pain is defined as “*an unpleasant sensory and emotional experience arising from actual or potential tissue damage or described in terms of such damage*” (1). Pain is a debilitating clinical condition and a major public health problem worldwide that incurs huge costs to society (2, 3). Depending on the nature and duration of the pain conditions, pain is usually categorized as acute or chronic, although the critical factors that increase the risk of transition from an acute to chronic state are still largely unknown (4, 5). Millions of patients suffer from acute pain each year with the vast majority of it being post-surgical (80-86% of patients following surgical procedures) types of severe acute pain, followed by pain associated with trauma, orthopedic, cardiac, and abdominal conditions (5, 6). In 2011, at least 100 million American adults reported chronic pain conditions outside of the acute pain conditions described above (7). Chronic pain is a pathological state that occurs on its own or accompanies several major disease conditions (e.g. obesity, AIDS, diabetes), the most common of its kind, caused by damage to neurons, is called neuropathic pain (8, 9).

Acute and chronic pain are different clinical conditions with distinct underlying pathophysiological mechanisms and characteristics (9). Acute or physiological pain is nociceptive by nature and caused by unavoidable tissue damage or injury (noxious

stimulation) that activates the peripheral sensory afferents and/or the peripheral inflammatory responses (activated immune system under some conditions) leading to protective behaviors that manifest either immediately (withdrawal reflex) or within a specified time frame (recovery and healing period) (1, 10). When pain persists beyond the normal recovery period, it is said to be maladaptive and pathologically chronic. A large portion of the preclinical animal data provides evidence for mechanisms underlying the transition from acute to chronic pain which is mainly inflammatory and neuropathic pain, including altered sensitivity of peripheral sensory neurons (*'peripheral sensitization'*) (11, 12), neuroplastic changes in the dorsal horn of the spinal cord and higher brain centers (*'central sensitization'*) (13) (see (14, 15) for a detailed review). Supporting these neuroadaptations are animal studies that have shown upregulation of the immune and glial cell activities at the peripheral site of injury, dorsal root ganglia, and the dorsal spinal cord (1, 16). These neuroadaptations subsequently underlie the characteristic behavioral features of chronic neuropathic pain such as the hypersensitive responses to innocuous stimuli termed "allodynia", exaggerated responses to noxious stimuli termed "hyperalgesia" or responses arising "spontaneously" (14, 15, 17, 18). Allodynia and hyperalgesia are classified as "stimulus-evoked" pain as they are behavioral signs that result from thermal, mechanical, or chemical stimulation (18) and can be easily measured in laboratory animals as reflexive withdrawal responses to the applied stimulus (19). Spontaneous pain or "stimulus-independent" pain are described as shooting, burning, or stabbing sensations of on-going discomfort, those that may be persistent (18, 20, 21), bothersome, a difficult aspect of chronic pain to treat (see (22, 23) for a detailed review), and generally more difficult to assess in non-verbal animals (24).

Therefore, in addition to a sensory-discriminative dimension, chronic pain is accompanied by severe motivational-affective (negative subjective experience), cognitive, and emotional dimensions that drastically alter quality of life and functioning, making it a multi-faceted and complex phenomenon (see (25-27) for reviews).

Prescription opioids, primarily μ opioid receptor agonists such as naturally occurring opioids – morphine, semi-synthetic derivatives (e.g., oxycodone), and synthetic opioids (e.g., fentanyl), are the mainstay treatment for the clinical management of moderate-to-severe acute pain (28-30) and chronic cancer pain ((31, 32), see (33, 34) for reviews). It is well-established that systemically administered prescription opioids produce analgesic effects by acting on μ opioid receptors located supraspinally in cortical and sub-cortical regions, mid-brain/brainstem regions controlling descending nociceptive processing, spinal regions of the central nervous system (see (35-37) for reviews), and at peripheral sites (38, 39). The extensive distribution of μ receptors all along the neuraxis inclusive of regions such as amygdala, thalamus, somatosensory cortex, periaqueductal gray (PAG), and rostral ventromedial medulla (RVM) contribute to the ability of opioids to modulate both the sensory and affective dimensions of pain states (36, 37, 40). In addition to potent analgesic properties, prescription opioids in clinical care often present with severe adverse effects including nausea, constipation, sedation, pruritis, respiratory depression, and tolerance ((41, 42), see (43) for review). More importantly, these drugs have rewarding properties that are known to be mediated by activation of the μ receptors located in the mesolimbic and nigrostriatal reward circuitries (44, 45), and subsequent interaction with the dopaminergic system that plays a vital role in mediating drug reward and addiction-related behaviors (46). Therefore, the use of prescription opioids for the

long-term management of non-malignant chronic persistent pain has been debatable (3), several controversies exist primarily regarding safety concerns relating to opioid dependence and addiction in clinical settings (47-49).

Sex Differences

Pain Sensitivity, Opioid Analgesia, and Opioid-Reward

Concordant data from clinical and human laboratory studies support the finding that women are more likely to report pain (e.g., fibromyalgia, irritable bowel syndrome), experience increased sensitivity to multiple pain modalities with the magnitude of difference in sensitivities between males and females varying across stimulus modalities: e.g., magnitude of difference in sensitivities to heat pain or pressure pain stimuli being greater than to ischaemic or cold pain stimuli; and lower threshold and tolerance to noxious stimuli than men (see (50-53) for reviews). Similar observations have been noted in the animal literature. Particularly in rodents, females reveal increased sensitivities and lower threshold to various modalities of nociceptive stimuli including thermal (54), mechanical (55, 56), chemical (57), and inflammatory pain (58), although differences are observed across different species, genotypes, and the nature of injury and types of pain models employed. Sex differences in opioid analgesia have also been rigorously studied. While morphine appears more efficacious in women than men consistently in clinical studies that are purely based on patient controlled analgesia (PCA) measures (59), human experimental studies reveal either an increased effectiveness for morphine in females (60) or no sex difference (61, 62) across different pain models. Further, the findings with other μ -receptor agonists are far less consistent (see (63, 64)

for reviews). The animal literature contrasts with the human findings in that the majority of the studies in rodents demonstrate increased μ opioid analgesia in males, although sometimes this finding varies depending on the pain model, genotype, and relative efficacies of the μ opioid agonists (55, 65, 66). Multiple factors underscore sex differences observed in animals and humans with respect to pain sensitivity and opioid analgesia. Much of the animal studies have elucidated the role of biological factors including the role of gonadal hormone functioning and its interactions with the endogenous opioid system at the neuroanatomical level. Other roles for genetic factors, drug-related pharmacokinetic and pharmacodynamic effects (see (67, 68) for reviews). However, an interplay between biological factors, psychological and socio-cultural elements are likely to make the sex-difference phenomenon more complex in humans (see (69, 70) for reviews).

In contrast to opioid-induced analgesic effects, sex differences in the abuse liability of μ opioid agonists have been studied to a lesser extent. In normal healthy volunteers, μ opioid agonists such as morphine and oxycodone produce greater positive subjective effects (“high”, “drug liking”) in males and greater unpleasant negative subjective effects (e.g. nausea, dysphoria, “spaced out” feelings) in females (71-73). In contrast, in a recent study in sporadic prescription opioid abusers, females reported higher ratings of “high”, “liking”, and “street value” for oxycodone administered intranasally (74). Behavioral evidence in animals is in line with the human studies where female rodents demonstrated increased sensitivity to the rewarding and reinforcing effects of μ opioid agonists such as morphine, heroin, and fentanyl using conditioned place preference and self-administration models (i.v. and oral) (75-80). Rudimentary

information exists regarding the mechanisms underlying sex differences in opioid-induced reward and reinforcing properties. For example, one study has implicated the modulatory activation effect of the gonadal hormone estrogen in the increased propensity for acquisition and greater consumption of heroin in female rats (81). Therefore, given the complex and overwhelming clinical and preclinical literature surrounding sex-differences in pain sensitivity, opioid analgesia, and opioid reward, it is compelling to determine the impact of sex/gender in the propensity to opioid reward as a function of pain.

Prescription Opioid Abuse

An alarming rise has been noted over the last two decade in the non-medical use of prescription psychotherapeutics in the United States with the prescription opioids being one of the top on the list (82-84). A status update on the non-medical use and abuse of prescription opioids almost equaling that of cocaine and heroin abuse was made by Zacny and colleagues in 2003 (85), and ever since that period, this problem has burgeoned (86, 87). Particularly, the results of the 2010 National Survey on Drug Use and Health had revealed that an estimated 22.6 million (8.9%) Americans, age 12 or older, were current (past month) illicit drug users, of which 5.1 million had used prescription pain relievers (88). Since the mid to late 1990's, a plethora of evidence supports a concurrent surge in the sales of opioid prescriptions and deaths resulting from opioid overdose (84, 89). Specifically, the estimated emergency department visits involving use of opioid pain relievers increased by 111% from 2004 to 2008 (Drug Abuse Warning Network report, 2010) (90). Among all the prescription opioids, most opioid

overdose-related deaths have been positively correlated with the increase in sales and prescribed use of hydrocodone- and oxycodone-containing therapeutics followed by methadone and morphine ((90), see (84, 91) for review). Among non-medical prescription opioid users aged 12 or older in 2009-2010, a majority (55%) reported obtaining the drug from friends or relatives for free (who had been prescribed opioids), 17.3% reported getting the drug directly from one doctor, while 4.4% obtained the pain relievers from drug dealers or strangers (88). Converging evidence suggests that the primary reasons driving the escalation of opioid prescribing by physicians have been the problem of under-treatment of pain as well as insufficient knowledge of the long term safety and efficacy of using prescription opioids for non-malignant chronic pain. Despite this rising problem, the factors including the role of pain in the propensity to misuse and abuse of prescription opioids remain poorly understood.

Prevalence of Prescription Opioid Abuse Among Pain Patients

Clinical management of chronic pain poses a major challenge as repeated use of therapeutic opioids inevitably produces tolerance and dependence that overlap with, and are difficult to dissociate from addiction-related phenomenon (92, 93). Lack of reliable empirical data limit the conclusions about the true incidence of prescription opioid abuse among chronic pain patients receiving chronic opioid analgesic therapy. The prevalence of opioid addiction in chronic non-malignant pain patients is estimated to vary broadly depending on the survey population and the criteria established for defining opioid addiction across studies (94-99). For example, Fishbain et al. (94) collated data from articles reporting drug misuse diagnoses, including drug abuse and dependence in chronic

pain patients and noted a prevalence percentage in a range of 3.2-18.9%, while alternative studies using urine toxicology and aberrant drug-related behavioral measures (including lost or stolen prescriptions, consumption in excess dosages than prescribed, additional clinic visits without appointments, multiple drug intolerances, and frequent telephone calls to seek more medication) in patients receiving long-term opioid therapy for chronic pain identified a prevalence percentage as high as 43% (96).

Many factors have been identified as predictors of prescription opioid misuse or abuse among pain patients, including psychological, sociodemographic, drug-related, and genetic (see (87) for review). Specifically, several studies reported past alcohol or polydrug use (100, 101) and co-morbid psychological issues, particularly depression (102, 103) as strong predictors of opioid misuse amongst those receiving opioid therapy. Illustration 1 depicts the complex interaction patterns between potential factors that may contribute to increasing the propensity of chronic pain patients to change their use of opioids from a pattern of normal therapeutic to compulsive use. Specifically, the co-occurrence of genetic factors, psychological illness(es), and chronic pain may pose the greatest risk to prescription opioid abuse in this population. Prospective cohort studies have also reported on gender-specific risk factors, including women using their prescribed opioids to cope with negative affect and/or co-morbid psychiatric distress compared to men (104-107), and sex-specific aberrant prescription use behaviors (e.g. women hoarding unused medications and men tampering with their medications [crushing and snorting pills]) (106). The Center for Disease Control and Prevention recently highlighted the increased percentage of death among women from opioid pain relievers and the need for

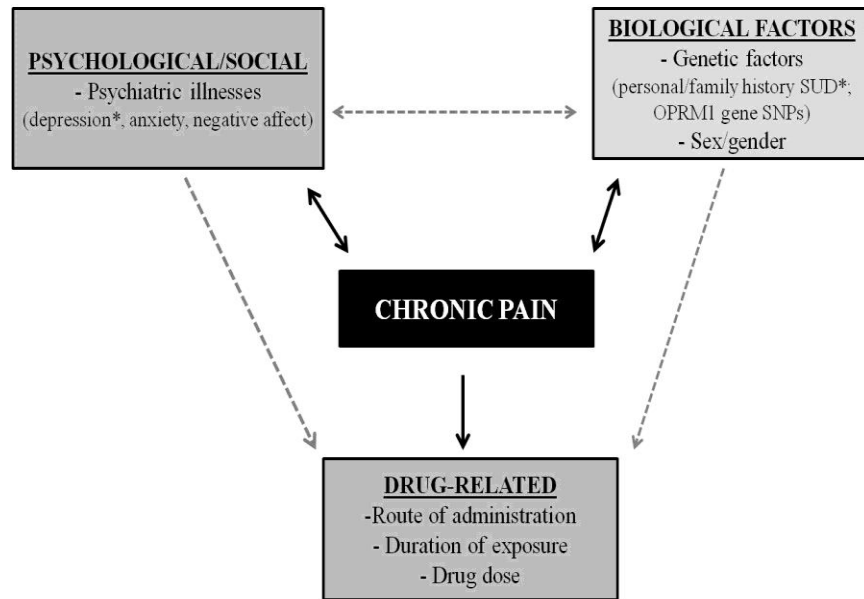


Illustration 1. Illustration of risk factors predicting predisposition to prescription opioid abuse among chronic pain patients (collated from review by Sehgal et al. (87)). *, strong predictors that when co-morbid with chronic pain. SUD: substance use disorders; SNPs: single nucleotide polymorphisms; OPRM1: human μ -1 receptor gene.

stringent drug monitoring programs for women in pain clinics (108). Taken together, these reports suggest that more systematic studies are warranted to better understand the neuropsychopharmacology and biological basis of opioid addiction as a function of pain in order to aid the development of more efficacious gender-specific analgesics with minimal side effect profiles.

Addiction-Related Behavioral Effects of Opioids as a Function of Pain

Clinical and Preclinical Update

Healthcare providers in the United States are faced with the two major problems: undertreatment of pain and prescription opioid misuse/abuse. Further, it is difficult to anticipate the risk versus benefit of prescribing opioids for the management of chronic

non-malignant pain. The concurrent assessment of analgesia and abuse potential of prescription opioids in the context of pain remains a challenge, mainly due to the difficulty of being unable to dissociate drug seeking for pain relief from drug seeking for abuse-related reasons in this patient population. One consequence of this major clinical concern has been a growing research focus on identifying potential risk factors that may contribute to increased opioid abuse (as mentioned above). Eventually, it may be possible to identify “at-risk” patients so that appropriate treatment interventions can be implemented (see (87) for extensive review). Other research efforts have focused on investigating the addiction-related behavioral effects of prescription opioids as a function of pain and/or other identified risk factors. In this regard, some studies have shown that the subjective effects (positive and negative effects) of opioids (dependent on type of opioid tested) are attenuated in the presence of acute experimentally-induced pain (109-111), and that opioids function as reinforcers primarily by producing pain relief in non-abusers (self-administration/choice behavior) (73, 112). However, the pharmacodynamic effects of opioids appear resistant to modulation by pain in opioid-abusers (73, 111). While studies including different human subject groups do reveal important information, it is often difficult to attain homogeneity in these subject groups, given that human subjects have diverse histories, such as illicit drug use patterns/complex addiction profiles, types and degree of chronic pain, psychiatric episodes, and socio-demographic variations. Therefore, given the co-morbidity and complexity of profiles in humans, data pertaining to the contribution of individual factors alone (e.g. chronic pain alone, sex/gender) in the propensity to prescription opioid misuse is limited.

Animal studies, using relevant preclinical models, allow for the evaluation of the role of purely biological factors (e.g. pain, sex/gender, genetic) independent of psychosocial and epidemiological contribution in the modulation of the reward-related behavioral effects of prescription opioids using relevant preclinical models. Recent evidence suggests that neurobiological changes occur both in the pain-related and reward-related brain regions with the development of persistent chronic pain (113-115), which stands to reason that the reward-related behavioral effects of opioids may be altered in the presence of pain. This has urged a growing body of research to address this question by incorporating different animal behavioral models of addiction and pain, predominantly in rats and only in male animals (113, 116-119). The general consensus across studies has been that the pharmacology of opioids is altered in the presence of pain and that pain-relief may be the primary motivation to maintain opioid-seeking and self-administration behaviors as measured in rodents (117, 118). However, the understanding of whether pain directly modulates the abuse potential of prescription opioids (different aspects of opioid reward including subjective effects and reinforcing efficacy) and the role of sex /gender [gained a great deal of attention recently in this field (see (120) for review)] in the interaction between pain and opioid reward, yet are largely unknown. These studies will be key to understanding the neurobiology of the interaction between pain and opioid reward and provide important preclinical tools to assess sex-specific pharmacological treatment interventions with low abuse potential for the clinical management of pain.

Preclinical Drug Abuse Models

A number of preclinical drug abuse models have evolved since the early to mid 1960s to evaluate and predict the abuse liability of drugs in laboratory animals (121), including drug self-administration, drug discrimination, and conditioned place preference (CPP). These models have proven valuable in evaluating the rewarding/reinforcing behavioral effects of drugs (121-123) and the neurobiology of drug addiction (124, 125). Conditioned place preference is based on a Pavlovian conditioning phenomenon where, after repeated pairing of a conditioned stimulus (particular environment) with an unconditioned stimulus (drug or saline injection), the preference of an animal for the drug or saline-paired environment is measured. Rewarding drugs typically cause the animals to spend a greater amount of time in the drug-paired environment, serving as an indirect technique to measure drug seeking and rewarding/motivational effects of drugs mediated by the reward circuitry. However, the CPP procedure has been criticized for, lacking face validity as a model of drug reward in humans (see (122, 126) for a detailed review).

Models of intravenous and oral drug self-administration procedures provide adaptable techniques to measure the ability of drugs to function as reinforcers and examine the relative reinforcing efficacy of drugs in terms of variables such as the preferred rates and patterns of drug consumption, and variables representative of drug-taking and drug-seeking behaviors using selective schedules of drug reinforcement in laboratory animals (see (123, 127-129) for reviews). For example, simple fixed ratio (FR) schedules where the animals receive drug infusions after completion of a fixed number of behavioral responses are dependent on the rates of responding and are valuable in the initial screening of drugs to function as reinforcers, however, the schedule

is relatively less sensitive to detecting the relative reinforcing efficacy of drugs and modulation by pharmacological and/or other manipulations (e.g., intracerebral injections and hormonal changes) (128, 130). In comparison, progressive ratio (PR) schedules of reinforcement where the response requirements to earn each subsequent injection of drug by the animal is increased and dependent on the effort the animal exhibits to earn drug infusions (defined as "breakpoint"), is a sensitive measure of the relative reinforcing efficacy and motivational values of drugs of abuse, and sensitive to pharmacological interventions (128, 130). Also, as PR measures the persistence in responding maintained by the drug (incentive-motivational value) and is mostly independent of the rate of responding, it represents a model of drug craving in humans (123, 127). Further, extinction responding obtained by removal of the drug or replacement of a neutral injection (saline) in place of the drug following extensive sessions of drug reinforcement also provides an alternative measure of drug craving in terms of the persistence of behavioral responses elicited by the animals in the absence of the drug (drug-seeking behavior) (127).

Drug discrimination is a paradigm that was devised to measure the behavioral effects of drugs mediated by their interaction with specific receptors, enzymes or ion channels in the CNS, and can serve as cues or interoceptive stimuli in the process (121, 131). It has been recognized that the stimulus properties of drugs may correspond loosely to the subjective effects of drugs or the underlying neuropharmacological/neurochemical basis associated with the discriminative properties of drugs (132). Previous studies provide evidence for the subjective effects of the drugs assessed in animals using drug discrimination procedures to be analogous to the

subjective effects of the same drugs in humans (133-135). This has facilitated the use of drug discrimination as a powerful preclinical tool for the screening of drugs for their abuse liability and understanding the neuropharmacological basis of their effects. Also, drug discrimination data provide a different dimension of a drug's effects than does drug self-administration data, as the behavioral effects of drugs subserving the discriminative stimulus properties do not necessarily overlap with the positive reinforcing effects of drugs of abuse as determined using self-administration paradigms (121). Overall, both drug discrimination and drug self-administration models have good predictive validity and display high concordance with clinical abuse liability measures, namely reports of positive subjective effects (e.g., 'euphoria', 'liking', 'high') and abuse of drugs by humans, particularly opioids and psychostimulants (121, 129, 136).

Therefore, study of different drugs of abuse, particularly opioids, as a function of pain using different behavioral procedures (as mentioned above) can provide a valuable approach to assess different aspects of opioid reward, that are likely mediated by different underlying processes.

Current Aims

The overall objective of the current thesis was to evaluate the role of acute *versus* chronic pain-states in the propensity to prescription opioid reward in C57Bl/6 mice and to determine if these variables varied by sex. In order to attain this objective, the current studies incorporated two putative mouse pain models that were each induced by a chemical modality: acute pain - acetic acid-induced visceral nociception; chronic pain - paclitaxel-induced peripheral neuropathy. The current studies were conducted in the

C57Bl/6 mice, an inbred mouse strain previously reported to be more sensitive to drug-induced rewarding effects (e.g. morphine, cocaine) compared to other inbred mouse strains (e.g. DBA/2J, SWR/J, BALB/c) (137-139). These studies involved the comprehensive and concomitant assessment of different aspects of prescription opioid reward using specific behavioral models of addiction to answer an important question of whether and how the above-mentioned types of pain modulate the positive subjective effects and the addiction potential of prescription opioids dependent on sex/gender.

In order to assess the addiction-related behavioral effects of opioids in the presence of acute and chronic pain, mouse pain models representative of brief and long-term hypersensitive human pain conditions were chosen. In particular, procedures were selected that do not produce generalized physical dysfunction, loss of body weight, or motor impairments that may otherwise prevent the animals from performing the required operant behavioral tasks. Acute acetic acid-induced chemical nociception (140) was incorporated in the acute pain studies primarily because it is a clinically relevant acute pain model (e.g. acute abdominal visceral pain conditions such as irritable bowel syndrome) (141, 142) in comparison to several commonly used animal models of acute pain (e.g. hotplate, tailflick, etc., where brief noxious stimuli are transiently applied to measure the withdrawal reflex) (19). In addition, acetic acid-induced chemical nociception does not produce overt signs of behavioral debilitations or peritonitis upon repeated, regular intervals of intraperitoneal administration as previously noted in our laboratory (Neelakantan et al., under review).

Similar to the acute chemical-induced nociceptive pain model, a chemical (chemotherapeutic drug)-induced model of chronic pain was used in the chronic pain

studies. Peripheral neuropathy is a common dose-limiting adverse effect accompanying the use of several potent anti-cancer drugs, including paclitaxel (143), and often times cancer survivors receiving chemotherapy treatment, especially breast cancer patients with paclitaxel treatments, develop long-term persistent neuropathic pain (20). When administered intraperitoneally in rodents, paclitaxel is a non-invasive, non-surgical technique to induce changes to sensory neurons that typically results in behavioral manifestations characteristic of chronic neuropathic pain conditions (e.g. allodynia, hyperalgesia) (144, 145). Paclitaxel-induced chronic peripheral neuropathy has predominantly been used preclinically to understand the mechanisms underlying chemotherapy-induced neurotoxicity and evaluate potential pharmacological agents that could reverse or prevent the development of peripheral neuropathy (144, 146). Our laboratory has previously demonstrated paclitaxel-induced persistent increases in sensitivity to mechanical and cold stimuli in C57Bl/6 mice that lasts for over 8 weeks, representing a robust mouse model of putative chronic pain (147, 148). Therefore, paclitaxel-induced peripheral neuropathy was used as a chronic pain to address the questions concerning the primary objective of the current study.

The first set of studies involved the comprehensive assessment of sex differences in the sensitivities and pharmacological effects of opioids in modulating the discriminative-sensory dimensions of pain (visceral nociception and allodynia) in mice using the two putative models of pain outlined above. Based on the fundamental framework built around the two models of pain in male and female mice, the second and third studies evaluated the reward-related behavioral effects of prescription opioids as a function of pain in male *versus* female mice using three behavioral models of drug

conditioning, namely drug discrimination, conditioned place preference, and intravenous drug self-administration. While morphine, a prototype μ -opioid receptor agonist and commonly used prescription opioid for the clinical management of pain was the primary drug tested, oxycodone, an alternative commonly prescribed and abused opioid (149, 150) was incorporated in parts of the following studies to compare the two opioids. The rationale for testing morphine and oxycodone were not only based on their high abuse potential, but also that morphine and oxycodone display differential pharmacokinetic profiles [e.g., contribution of morphine's metabolite to its analgesic effects (151) compared to oxycodone's effects that may be primarily mediated on its own (largely based on single dose studies – see (152, 153))], and differential pharmacological profiles in specific animal models of pain (154, 155).

Based on anecdotal reports suggesting decreased likelihood of prescription opioid misuse/abuse in an acute pain setting (49), increased risk of prescription opioid abuse and dependence in a chronic pain setting (96), and the extensive preclinical and clinical sex differences literature demonstrating significant sex differences in the propensity to pain and pharmacological effects of opioids (less clear sex-differences in humans) (120), it was predicted that the reward-related behavioral effects of prescription opioids will be differentially altered in the presence of acute versus chronic pain and will be dependent on sex. More specifically, the studies were designed to test the following hypotheses: i) the presence of acute acetic acid-induced nociception will attenuate the discriminative stimulus effects but not alter the reward circuitry-mediated behavioral effects of prescription opioids in C57Bl/6 mice; ii) the presence of chronic paclitaxel-induced peripheral neuropathy will attenuate the discriminative stimulus effects and dose-

dependently increase or decrease the reward circuitry-mediated behavioral effects of prescription opioids in C57Bl/6 mice; iii) the modulation of the reward-related behavioral effects of prescription opioids as a function of pain will be more pronounced in male but not female mice since there is preclinical evidence for decreased sensitivity of female rodents to the analgesic effects of opioids and increased sensitivity to opioid-induced reward (156).

Taken-together, these studies will highlight the different aspects of prescription opioid-reward such as the conditioned rewarding, reinforcing efficacy, and discriminative stimulus effects (subjective effects) [in terms of drug-related variables (dose, opioid type)] as a function of differential pain states (acute *versus* chronic) in each sex.

CHAPTER 2
PUTATIVE CHEMICAL-INDUCED PAIN STATES AND OPIOID ANALGESIA
IN MALE AND FEMALE C57BL/6 MICE

Rationale

Acute pain is the most common symptom for which patients seek medical care and chronic pain, a transitioned long-term state from acute pain that is the leading cause of disability in the United States (1). In the last 20 years, an expansive literature has established sex-differences in responses to pain and analgesics in humans. Consistent clinical and experimental human laboratory observations indicate that many chronic pain syndromes are more prevalent among women, and women report increased severity and frequency of pain to multiple pain modalities (50-53, 120, 157). Sex differences in opioid analgesia, specifically μ opioid receptor agonist analgesia have been mixed between the clinical and experimental pain studies, the results being dependent on the pain model and stimulus modality employed in these studies (59, 64, 70). For example, Miaskowski et al. (59) reported women to consume less opioids in postoperative settings suggestive of increased sensitivity to opioid analgesia compared to men. While similar results have been noted in experimental studies employing electric or cold pressor models (137, 158), no sex differences were observed using the pressure pain model (158).

The vast majority of preclinical studies have demonstrated increased μ opioid analgesia (specifically morphine) in male relative to female rodents using well established models of acute nociception, a majority of the studies using thermal assays, fewer chemical and mechanical paw withdrawal models (159). The results of these

studies are diametrically opposite to that in humans and dependent largely on the type and efficacy of opioid agonists tested so that an inverse relationship between analgesic potency and relative efficacy of opioid agonists exists in males (66). More recent studies have extended these findings to chronic pain models in rodents (e.g. arthritic, neuropathic, and inflammatory pain) where female rats displayed increased susceptibility to the development of persistent pain and reduced sensitivity to opioid anti-allodynic effects (160-162).

Preclinical studies provide evidence for the role of biological factors as potential mechanisms underlying sex differences in pain sensitivity and opioid analgesia. The most well-studied factors are the organizational and activational effects of gonadal sex hormones on the neural circuitry underlying pain transmission (67, 163-165). The role of estradiol alone has been extensively studied. Specifically, female animals display increased sensitivity to noxious thermal, mechanical, and chemical stimuli during the high estradiol cycle phase (58, 162, 166, 167) and decreased sensitivity to the antinociceptive effects of μ opioid agonists (168-170). In contrast, gonadally intact adult males display increased sensitivity to the antinociceptive effects of μ opioid receptor agonists (170, 171). Recent research has expanded to integrate genetic factors in pain-related traits in animals and humans (120, 172, 173). Transgenic mouse models are used to study the different pain-related phenotypes dependent on strain/genotype and potential sex/genotype interactions in these effects (65, 174-179). Although the preclinical sex difference literature has been steadfastly growing, it is important to note that the majority of these reports are contributed by studies using acute thermal pain tests in rats (e.g. tail-flick, hotplate, tail-withdrawal), with only recent expansion of this literature to include

other types of more clinically relevant acute and chronic pain models in rodents (156, 159).

The aims of this study were to: i) systematically assess the baseline behavioral responses and sensitivities of male and female C57Bl/6 mice to two putative chemical-induced pain states using the acute acetic acid-induced chemical nociception and chronic paclitaxel-induced peripheral neuropathy; and, ii) determine the sensitivities of male *versus* female mice to opioid-induced antinociceptive and anti-allodynic effects using these two mouse models of pain. Acetic acid-induced nociception is a well-established model of acute visceral pain in rodents. It is traditionally used as an acute preclinical rodent analgesia test for screening potential analgesic compounds (180-183). While the effects of morphine are well-established in this acute pain test in rodents (183-187), the effects of other μ opioid agonists such as oxycodone, and potential sexual dimorphism of these opioid effects are not fully known. Paclitaxel-induced peripheral neuropathy is a preclinical rodent model of chronic pain primarily used to determine the mechanisms underlying chemotherapy-induced neurotoxicity and to evaluate the effectiveness of potential preventative measures in rodents (144, 146). For example, studies have increasingly focused on and demonstrated a few potential mechanisms of paclitaxel-induced damage to peripheral sensory neurons such as neuronal mitochondrial and axonal transport dysfunction, altered signal conduction, and distal axonal degeneration (145, 188-190). However, fewer studies have comprehensively assessed and/or established potential sex differences in pain behaviors and opioid-induced behavioral effects using the above-mentioned putative chemical-induced models of acute and chronic pain in rodents (167, 191).

Methods

Subjects

Male C57Bl/6 mice weighing 20-25 g, and female mice weighing 15-20 g, and all mice 5-6 weeks of age, were purchased from Taconic, Farms, Inc. (Cranbury, NJ, USA) for the acetic acid (N=11, male; N=11, females) and paclitaxel experiments (N=90, males; N=104, females). Mice were group housed (groups of four) in plastic cages and allowed to acclimate to the temperature-and-humidity controlled animal facility for 3 to 7 days before the experiments began. Mice were housed under a 12 h light/dark cycle with lights off at 10.00 h, so that all experiments occurred during the dark phase of the diurnal cycle of the mice. Food and water were available *ad libitum*. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University and the Guide for the Care and Use of Laboratory Animals (Institution of Laboratory Animal Research, National Academy Press; Eighth edition, revised 2011).

Drugs

Morphine sulfate and oxycodone hydrochloride were generously donated by the National Institute on Drug Abuse (NIDA) drug supply program (Bethesda, MD, USA). Paclitaxel (dissolved in a 1:1 mixture of alcohol and Cremophor) was obtained as a 6 mg/mL concentration stock solution (Hospira, Inc., Lake Forest, IL, USA). Morphine, oxycodone, and paclitaxel were dissolved in 0.9% saline. Cremophor vehicle was made up as a 1:1:18 ratio ethanol:Cremophor:saline solution. A stock solution of 1 N acetic acid was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and diluted to 0.4%

concentration solution in sterile water. All injections were administered intraperitoneally (IP) in a volume of 0.01 ml/g of body weight.

Experiment 1: Acute Acetic Acid-Induced Nociception

Apparatus

The testing cages for the acetic acid experiments were separate Plexiglas observation cages that were similar in dimension to the cages in which mice were housed in the animal facility. Test sessions were recorded and the videos were subsequently scored by an observer blinded to the treatment conditions.

Procedure

The acetic acid-induced stretching assay was adapted and modified from Koster et al. (140) and used as model of acute chemical-induced visceral nociception. In this procedure, mice were injected intraperitoneally with acetic acid (0.4% concentration). The opioids were also administered IP in this assay similar to previous procedures reported in the literature (182, 192), and to maintain the behavioral procedure consistent across studies (compare with drug discrimination assay in chapter 3). This was a within-subject study procedure in which different groups of male and female mice were tested repeatedly once every week. During the first week, all mice were injected consecutively with saline (drug vehicle condition) and an intermediate 0.4% concentration of acetic acid on each side of the peritoneal cavity and then placed individually in separate Plexiglas observation cages. The acetic acid-induced stretching behavior, defined by body contortions and extension of the hind limbs, was videotaped for a period of 40 min

for each mouse and the videos were subsequently scored. Acetic acid-induced stretching behaviors were compared between male and female groups of mice.

During the subsequent weeks, different groups of mice received morphine (0.1-3.2 mg/kg) or oxycodone (0.1-1 mg/kg) (tested in ascending dose orders across weeks) consecutively followed by 0.4% acetic acid, two injections administered on either side of the peritoneal cavity to determine the effects of the two opioids in reversing the acetic acid-stimulated stretching behavioral responses in male and female mice. Consecutive administration of drug followed by acetic acid (0 min pretreatment) was chosen in the present study to directly compare these results with the behavioral studies discussed in chapter 3.

Data and Statistical Analysis

For the data analysis, the primary dependent variable of the acetic acid studies was the number of stretches scored in the observation period (40 min). Each mouse served as its own control and the drug effects on the acetic acid-induced stretches data were transformed as % decrease in stretches and calculated by the following equation:

$$\% \text{decrease in stretches} = [(\text{control (post-vehicle) number of stretches} - \text{test (post-drug) number of stretches}) / \text{control number of stretches}] * 100$$

A value of 0 was assigned if the mouse produced more stretches after drug administration when compared to the number of stretches after the corresponding vehicle administration (determined during the first week of testing as mentioned above). The % decrease in stretches calculated for each mouse was averaged into a group mean for each dose of the two opioids tested in different groups of male and female mice. The number

of stretches produced by acetic acid in male and female mice was compared using unpaired Student's t-test. One-way ANOVA with Dunnett's posthoc analyses were used to determine the significant differences between either doses of morphine or oxycodone and saline to reverse stretching behavior within each sex. Two-way ANOVAs were used to determine the effects of drug dose and sex, and the interaction between the two factors. Statistical significance was set at $P < 0.05$ for all analyses.

Experiment 2: Chronic Paclitaxel-Induced Peripheral Neuropathy

Apparatus

Paclitaxel-induced peripheral neuropathy was assessed using two behavioral measures in C57Bl/6 mice: cold and mechanical allodynia. For both behavioral measures, mice were placed inside individual Plexiglas compartments atop a wire grid floor held 20 cm above the laboratory bench top.

Procedure

Cold allodynia. Male C57Bl/6 mice (n=50) and female C57Bl/6 mice (n=64) were treated with Cremophor vehicle (ethanol:Cremophor:saline in 1:1:18 ratio) or paclitaxel (1, 2, 4, or 8 mg/kg) on a standard dosing regimen of days 1, 3, 5, and 7 (147). Mice were acclimated in the Plexiglas compartments for 15 min before starting behavioral measures. Cold allodynia was measured using the acetone drop test (193) where acetone (approximately 50 μ L) was squirted via a needle and syringe onto the plantar surface of the left hind paw of the mouse and time spent lifting, fluttering, or licking the hind paw was recorded for 60 s. Cold allodynia assessments were performed

at the beginning of the experiment prior to starting paclitaxel or vehicle administration and recorded as the baseline measure. Behavioral testing was performed twice every week for about 3 to 8 weeks after the start of paclitaxel dosing. Sensitivities of male and female mice to paclitaxel-induced cold allodynia were recorded and compared between groups.

Mechanical allodynia. Male and female C57Bl/6 mice (n=24 per group) were treated with Cremophor vehicle (ethanol:Cremophor:saline in 1:1:18 ratio) or paclitaxel (4 or 8 mg/kg) on a standard dosing regimen of days 1, 3, 5, and 7 (147). Mice were acclimated in the Plexiglas compartments for 15 min before starting behavioral measures. Mechanical allodynia was measured using the Von Frey filament assay (194), where monofilaments of varying forces (0.07-4.0 g) were applied to the mid-plantar surface of the right hind paw, with each application held in c-shape for about 6 s using the up-down method to determine the paw withdrawal threshold sensitivity. Mechanical allodynia assessments were performed at the beginning of the experiment prior to starting paclitaxel or vehicle administration and recorded as the baseline measure. Behavioral testing was performed twice every week for about 3 to 8 weeks after the start of paclitaxel dosing. Paclitaxel-induced increase in sensitivities of male and female mice to mechanical pressure test were determined and compared between control and treatment groups within each sex.

Effects of morphine on paclitaxel-induced mechanical allodynia. Separate groups of male and female C57Bl/6 mice (n=8 per group) were treated with paclitaxel (8 mg/kg)

or saline on days 1, 3, 5 and 7. Paclitaxel-induced mechanical allodynia was measured using Von Frey filament assay as explained previously, on days 2, 5, and 8 post first paclitaxel injection. Starting on day 11 corresponding to the peak allodynia period, separate groups of saline- and paclitaxel-treated mice (n=8 per group) received one injection per day of morphine for three consecutive days. Within each group, half the mice (n=4) received the lower dose (2.5 mg/kg), and the remaining half (n=4) received the higher dose (10 mg/kg) of either drug. Following the third injection on day 3 (30 min pretreatment), mice were tested for mechanical allodynia. Mice were again injected with this three-day regimen for the next two weeks with either ascending (5.0 and 10 mg/kg) or descending doses (5.0 and 2.5 mg/kg) of morphine (counterbalanced within groups). The doses for morphine were chosen from a previous report on morphine's effective and ineffective doses in rat studies using the same rodent model of chronic pain (195). A three-day morphine treatment was incorporated in the present study to directly compare these study results with the behavioral studies discussed in chapter 4, and to approximate a repeated opioid administration schedule used for the management of chronic pain conditions in humans (196). At the beginning of each week, prior to the start of drug dosing, mechanical allodynia measurements were recorded. Mechanical sensitivity prior to and following treatment with morphine (2.5-10 mg/kg) in different groups of saline- or paclitaxel-treated male and female mice were determined and compared.

Data and Statistical Analysis

Two-way ANOVAs (GraphPad Prism 5.0 Software, Inc, La Jolla, CA) with the factors of treatment and time were used to determine the effects of paclitaxel treatment on

cold and mechanical allodynia in male and female groups. Three-way (PASW Statistics 18.0) ANOVA with factors of treatment, time, and sex were used to examine the effect of 2 mg/kg (dose at which significant sex effect was noted) of paclitaxel between sexes. For the morphine effects on paclitaxel-induced mechanical allodynia, the dose response effects were analyzed within treatment groups in male and female mice using repeated measures one-way ANOVAs with Dunnett's posthoc tests (GraphPad Prism 5.0 Software, Inc, La Jolla, CA).

Results

Experiment 1: Acute Acetic Acid-Induced Nociception

An intermediate concentration of acetic acid (0.4 %) produced comparable nociception in male and female C57Bl/6 mice represented by significant increases in abdominal stretching responses as recorded from a 40-min observation period post-acetic acid administration (Figure 1). Although not statistically significant, female mice displayed a trend toward increased sensitivity to acetic acid-induced acute nociception compared to male mice as revealed by an increased stretching response [$t = 1.42$, $df = 20$, $p = 0.0855$]. The distributions of acetic acid-induced stretching behavior over two 20-min bins have been represented in Figure 2. It was noted that intraperitoneal administration of acetic acid resulted in a faster onset of stretching response in male mice (between 3 to 4 min post acetic acid injection) compared to females where the onset was noted between 9 to 10 min post-acetic acid injections (Figure 2). Also, acetic acid-induced stretching responses were observed to occur at increased frequency

(representative of peak effect period) between 10- to 20-min and 20- to 30-min time frames post-acetic acid injections in male and female mice, respectively.

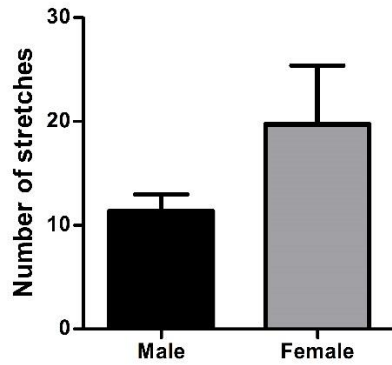


Figure 1. Acetic acid-induced stretching behavior in male (black bar) and female (gray bar) C57Bl/6 mice. Abscissa: sex of the C57Bl/6 mice. Ordinate: number of stretches recorded in a 40 min observation bin. All bars represent the mean number of stretches in the respective groups + SEM.

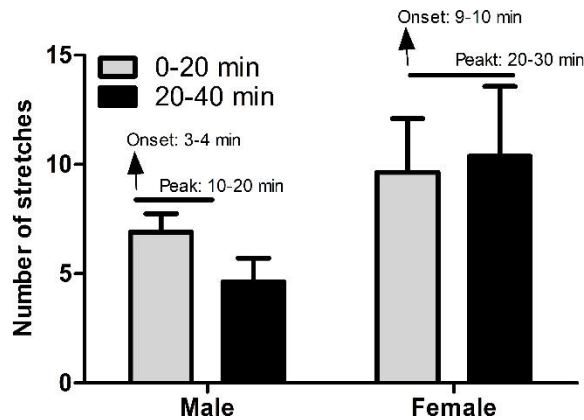


Figure 2. Acetic acid-induced stretching behavior in male and female C57Bl/6 mice during two 20-min bins. Abscissa: sex of the C57Bl/6 mice. Ordinate: number of stretches recorded in 0-20 min and 20-40 min observation bins post-acetic acid administration. All bars represent the mean number of stretches in the respective groups + SEM. The figure also represents the time of onset and peak acetic acid-induced stretching time frames in male and female C57Bl/6 mice.

Morphine produced dose-dependent effects in reversing acetic acid-induced nociception in male and female mice. One-way ANOVA within each sex revealed the first dose of morphine tested in this assay to be effective was 3.2 mg/kg in males and 0.1 mg/kg in females (at least a 32-fold increased potency in female mice). Further, a two-way ANOVA revealed significant main effects of sex [$F_{(1, 30)} = 12.91$, $p = 0.0012$] and dose [$F_{(2, 30)} = 5.57$, $p = 0.0088$], but no significant interaction [$F < 1.0$] between the factors (Figure 3, panel A). In contrast to morphine's effects, oxycodone produced greater antinociception in reversing acetic acid-induced stretching in male mice (3.2-fold increased potency), but the opposite effect in female mice (3.2-fold decreased potency) (see Table 1). One-way ANOVA within each sex revealed the first dose of oxycodone tested in this assay to be effective was 1 mg/kg in males and 0.32 mg/kg in females (at least a 3.1-fold increased potency in female mice) (see Table 1). A two-way ANOVA revealed a trend toward significance for the dose-effect [$F_{(2, 24)} = 3.26$, $p = 0.0558$], but no significant effect of sex or an interaction between the two factors [$F < 1.0$] (Figure 3, panel B).

Table 1. Summary of sex differences in the sensitivities of C57Bl/6 mice to acute acetic acid-induced nociception and opioid-induced antinociceptive effects.

Acute acetic acid-induced nociception: Sex differences in pain sensitivity			
Acute noxious stimulus	Behavioral endpoint	Males	Females
Acetic acid (0.4% conc., IP)	Abdominal stretches		Increased sensitivity (trend, p = 0.0855)

Prescription opioid-induced antinociception: Sex differences			
Acute noxious stimulus	Prescription opioid tested	Males (Effective Dose)	Females (Effective Dose)
AA (0.4%)	Morphine	3.2 mg/kg	0.1 mg/kg
	Oxycodone	1.0 mg/kg	0.32 mg/kg

Effective Dose: first dose of opioid tested that reversed acetic acid-induced stretching

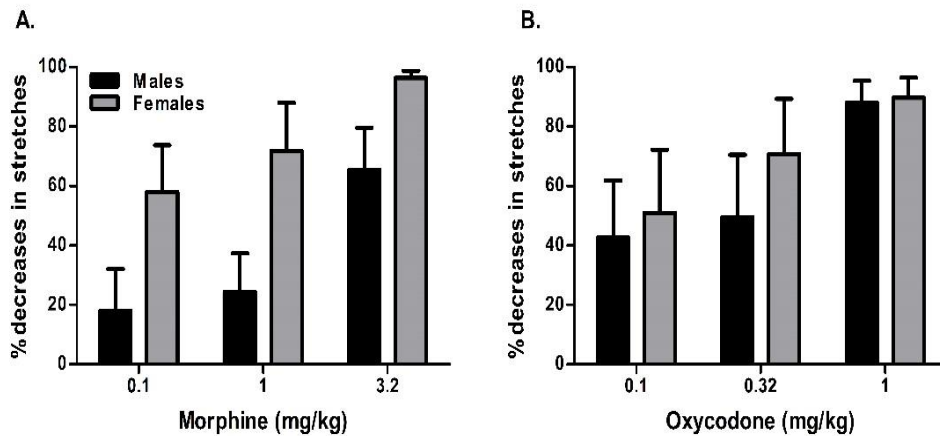


Figure 3. Effects of morphine (panel A) and oxycodone (panel B) in reversing acetic acid-induced stretching in male and female C57Bl/6 mice. Abscissa: dose of opioid, morphine (panel A) and oxycodone (panel B) expressed as mg/kg. Ordinate: % reduction in acetic acid-induced stretches. All bars represent the mean % reduction in stretches in the respective dose groups + SEM.

Experiment 2: Chronic Paclitaxel-Induced Peripheral Neuropathy

Paclitaxel produced allodynia (increased sensitivity to non-noxious cold and mechanical stimuli) in male and female C57Bl/6 mice. The lowest dose of paclitaxel tested (1.0 mg/kg \times 4 injections) produced significant cold allodynia only in female mice (Figure 4, panel A). A two-way ANOVA revealed significant main effects of sex [$F_{(1, 36)} = 10.3, p = 0.003$], time [$F_{(2, 36)} = 5.62, p = 0.008$], and a significant interaction [$F_{(2, 36)} = 6.61, p = 0.004$]. Four injections of paclitaxel at the 2.0 mg/kg dose produced cold allodynia of greater magnitude in female mice (Figure 4, panel B). A three-way ANOVA revealed a significant effect of sex (female) [$F_{(1,48)} = 7.51, p = 0.009$], treatment (paclitaxel) [$F_{(1,48)} = 10.8, p = 0.002$], and time (day 13) [$F_{(1,48)} = 5.71, p = 0.02$] but no interactions [$F < 1.0$]. These data also revealed that treatment with the Cremophor vehicle alone increased sensitivity to the cold stimulus in relation to the baseline behavior.

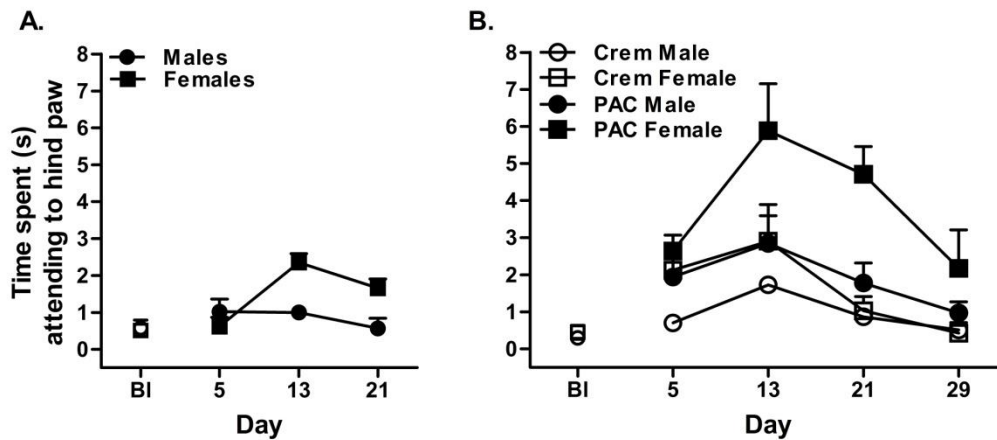


Figure 4. Effects of 1 mg/kg (panel A) and 2 mg/kg (panel B) paclitaxel (standard 4 injection dosing regimen days 1, 3, 5, and 7) on cold allodynia in male and female C57Bl/6 mice. Abscissa: Time points pre- or post-day 1 injection (BI = baseline). Ordinate: Time spent by mice lifting, fluttering, or licking hind paw after administration of 50 μ L acetone. Data points represent the mean time spent attending to the hind paw in seconds + SEM; $n = 6$ to 10 per group; Paclitaxel (PAC) or Cremophor (Crem).

Paclitaxel administration at the higher doses (4 and 8mg/kg) produced increased sensitivity to a cold stimulus, the effects being largely dose-independent and pronounced in female mice. Two-way ANOVA revealed significant main effects of treatment [$F_{(2,319)} = 8.37, p = 0.0003$], and time [$F_{(10,319)} = 2.99, p = 0.0013$], and a significant interaction between the two factors [$F_{(20,319)} = 1.72, p = 0.029$] in female mice (Figure 5, panel B), but no significant effect of either factors or any interaction in male mice [$F < 1.0$] (Figure 5, panels A). In contrast to cold allodynia effects, both male and female mice displayed increased sensitivity to paclitaxel (4 and 8 mg/kg)-induced mechanical allodynia (Figure 6, panels A and B). For mechanical allodynia, two-way ANOVA revealed significant main effects of treatment in females [$F_{(2,231)} = 20.34, p < 0.0001$] and males [$F_{(2,231)} = 44.66, p < 0.0001$], time in females [$F_{(10,231)} = 2.07, p = 0.0278$] and males [$F_{(10,231)} = 4.22, p < 0.0001$], and a significant interaction in males [$F_{(20,231)} = 1.68, p = 0.0376$] but not in females [$F < 1.0$]. Sex differences in the overall sensitivities of C57Bl/6 mice to paclitaxel-induced allodynia via different stimulus modalities are summarized in Table 2.

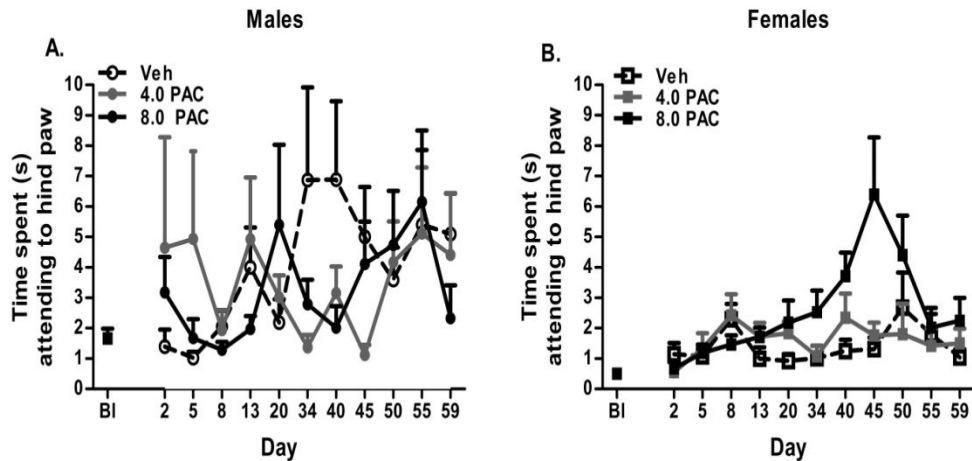


Figure 5. Effects of 4 mg/kg and 8 mg/kg paclitaxel (standard 4 injection dosing regimen days 1, 3, 5, and 7) on cold allodynia in male (panel A) and female (panel B) C57Bl/6 mice. Abscissa: Time points pre- or post-day 1 injection (BI = baseline). Ordinate: Time spent by mice lifting, fluttering, or licking hind paw after administration of 50 μ L acetone. Data points represent the mean time spent attending to the hind paw in seconds + SEM; n = 8 to 12 per group; Paclitaxel (PAC) or Cremophor (Veh).

In the last study, the dose-response effects of morphine in reversing paclitaxel-induced mechanical allodynia were assessed in male and female mice. Paclitaxel (8 mg/kg), compared to saline treatment, produced significant mechanical allodynia in females [$F_{(1,42)} = 12.42$, $p = 0.001$] and males [$F_{(1,42)} = 10.96$, $p = 0.0019$], significant effect of time in females [$F_{(2,42)} = 4.91$, $p = 0.0121$] and males [$F_{(2,42)} = 6.46$, $p = 0.0036$], but no interaction [$F < 0.1$] (Figure 7, panels A and C). In the saline treated groups, repeated measures one-way ANOVA revealed no significant effect of morphine alone on the mechanical sensitivity in male mice (Figure 7, saline panel B). In contrast, females displayed overall increased sensitivity to the mechanical stimulus when morphine was

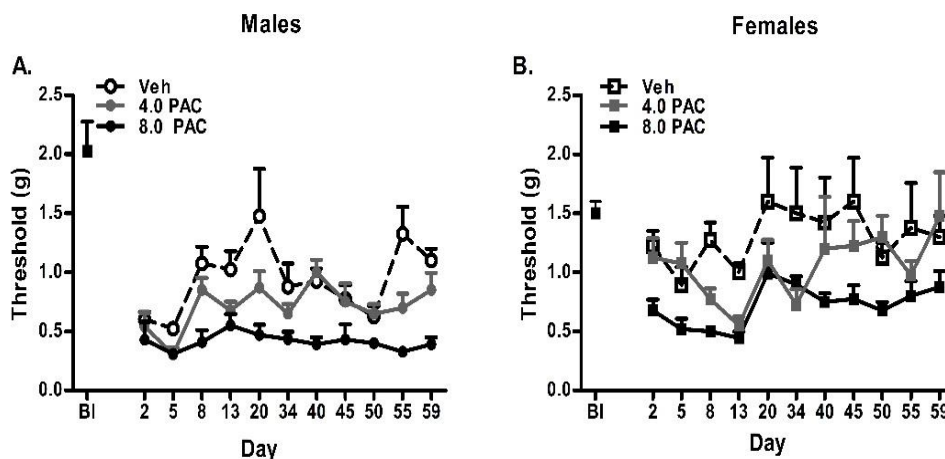


Figure 6. Effects of 4 mg/kg and 8 mg/kg paclitaxel (standard 4 injections dosing regimen days 1, 3, 5, and 7) on mechanical allodynia in male (panel A) and female (panel B) C57Bl/6 mice. Abscissa: Time points pre- or post-day 1 injection (BI = baseline). Ordinate: Threshold of sensitivity to the mechanical stimulus (g). Data points represent the mean paw withdrawal threshold in grams + SEM; n = 8 per group; Paclitaxel (PAC) or Cremphor (Veh).

administered on its own in the saline-treated groups ($p = 0.0215$), although no significant posthoc effect for any of the specific doses of morphine tested were observed (Figure 7, saline panel D). In the paclitaxel-treated groups, morphine produced dose-dependent reversal of mechanical allodynia in male and female mice (Figure 7, paclitaxel panels B and D). Male mice displayed increased sensitivity to morphine-induced anti-allodynic effects. The first dose of morphine tested to be effective in male mice was the lowest dose of morphine tested 2.5 mg/kg, while 5 mg/kg morphine dose was the observed effective dose in female mice (at least a 2-fold difference in potency between sexes) (see Table 2).

Table 2. Summary of sex differences in the sensitivities of C57Bl/6 mice to paclitaxel-induced cold and mechanical allodynia, and morphine-induced anti-allodynic effects.

Paclitaxel-induced allodynia: Sex differences in chronic pain sensitivity			
Paclitaxel dose	Allodynia modality	Males	Females
1 mg/kg, IP	Cold allodynia	No	Yes
2 mg/kg, IP	Cold Allodynia	No	Yes
4 mg/kg, IP	Cold Allodynia	No	Yes
	Mechanical Allodynia	Yes	Yes
8 mg/kg, IP	Cold Allodynia	No	Yes
	Mechanical Allodynia	Yes	Yes
Morphine-induced anti-allodynic effects: Sex differences			
Paclitaxel dose	Allodynia modality	Males (Effective dose)	Females (Effective dose)
8 mg/kg, IP	Mechanical Allodynia	2.5 mg/kg	5 mg/kg

Effective Dose: first dose of opioid tested that reversed acetic acid-induced stretching.

“Yes/No” indicates presence or absence of allodynia

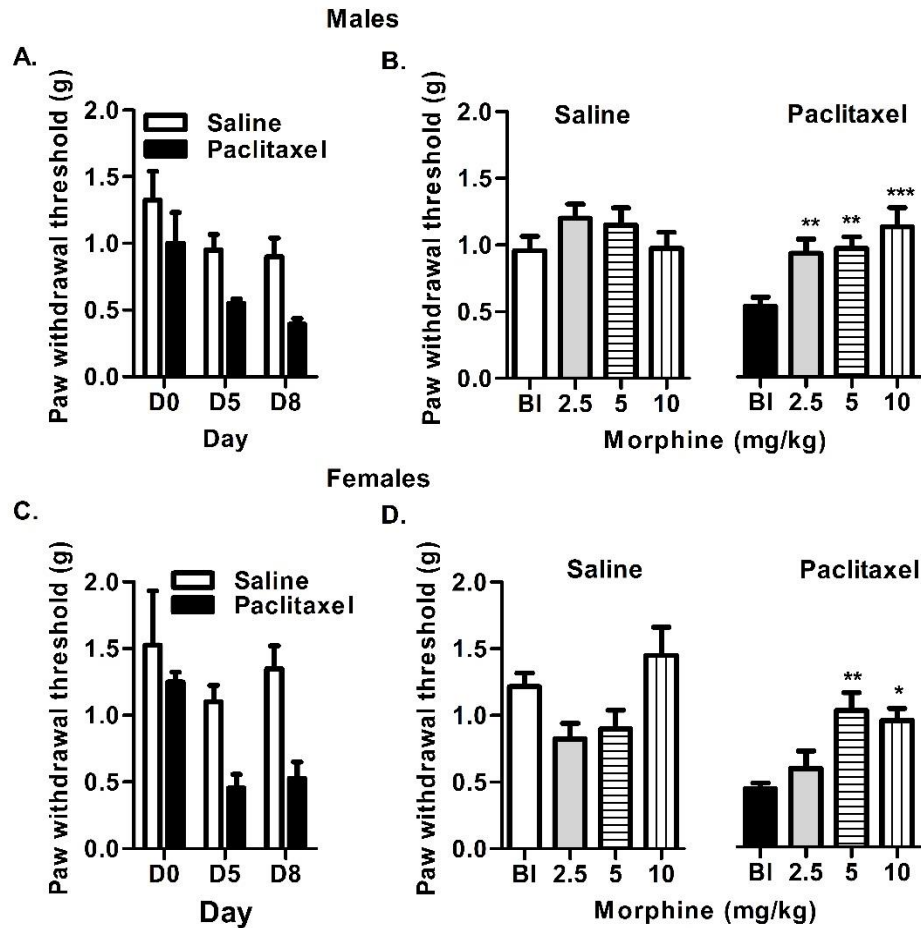


Figure 7. Paclitaxel (8 mg/kg)-induced mechanical allodynia (panels A and C) and effect of morphine (2.5 to 10 mg/kg) on paclitaxel-induced mechanical allodynia (tested between days 11-20 after start of paclitaxel administration) (panels B and D) in male and female C57Bl/6 mice. Abscissa: Time points pre- (D0) or post day 1 injection of paclitaxel (D5, 8: days 5 and 8) (panels A and C); morphine dose expressed as mg/kg (BI = baseline) (panels B and D). Ordinate: Threshold of sensitivity to the mechanical stimulus (g). Bars represent the mean paw withdrawal threshold in grams + SEM; n = 8 per group. Two-way ANOVA revealed significant main effects of treatment ($p < 0.01$) and time ($p < 0.01$) [panels A and C]; *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$ compared to the respective baselines (BI) in the paclitaxel-treated groups within each sex.

Discussion

The results of the current study confirmed the existence of sex differences in the sensitivities of an inbred mouse strain, C57Bl/6, to two putative chemical-induced pain states and opioid-induced antinociceptive and anti-allodynic effects. Female mice displayed increased sensitivities compared to male mice to the acetic acid-induced acute nociception and chronic paclitaxel-induced cold allodynia. Given the current experimental design and parameters, females revealed increased sensitivities to the antinociceptive effects of morphine and oxycodone in the acute acetic acid-induced nociceptive assay, and in contrast decreased sensitivity to the anti-allodynic effects of morphine in the chronic paclitaxel-induced mechanical allodynia test compared to male mice.

Consistent with previous studies, acetic acid administration induced an increased acute visceral nociceptive behavioral response in female mice (a trend in significant effect) compared to male mice in the present study (167, 197). However, discrepancies in the magnitude of sex differences between previous (significantly greater acetic acid nociception in females) and the current findings could be attributed to differences in the model of visceral pain (colorectal distension *vs* acetic acid in the present study), concentration of acetic acid (higher 2% *vs* 0.4% v/v in the present study), and species (rats *vs* mice in the present study). Increased sensitivity of female rodents to acute visceral nociception are in line with the clinical findings, where women are more predisposed to visceral pain syndromes (e.g. irritable bowel syndrome, chronic pelvic pain) (51, 198, 199). Interestingly, a sex difference in another characteristic of this acute nociceptive behavior were also noted in the present study - acetic acid administration

resulted in a quick onset (2 and 3 min) and early occurrence of peak nocifensive stretching behavioral response in male mice (peak effect between 10 and 20 min) compared to a delayed behavioral response (onset: 9 to 10 min; peak: 20 to 30 min) in female C57Bl/6 mice, a distinct observation not generally reported in the literature. The manner in which this time-dependent sex difference characteristic in the acetic acid stretching model may impact behavioral assessments, including antinociceptive effects of opioids (discussed below) and discriminative stimulus effects of opioids in the presence of this acute pain (discussed in chapter 3) have been discussed.

Morphine produced dose-dependent reversal of acetic acid-induced stretching behavior in male and female C57Bl/6 mice, but with significant differences in effectiveness between sexes. Specifically, female mice displayed increased sensitivity to morphine's antinociceptive effects compared to males (effective dose within the dose range tested, F: 0.1 mg/kg > M: 3.2 mg/kg). The present findings strikingly contrast with the results of previous studies in rodents in two ways: 1) the majority of studies in male rodents have shown increased effectiveness of morphine at lower doses using the acute chemical nociceptive test (potency ranging between 0.32 and 1 mg/kg) (183-187); 2) sex difference reports using chemical and thermal acute pain tests have uniformly reported increased effectiveness of morphine in males (M > F) (187, 200-202). The primary experimental parameter separating the previous studies and the current study is the time after administration of the opioid when acetic acid was injected. Specifically, morphine was administered (either subcutaneously or intraperitoneally) as a pretreatment 15 to 45 min prior to acetic acid in the previous studies. With this time point usually corresponding to the peak drug effect in both sexes, the magnitude and direction of sex

difference ($M > F$) remained the same across studies. However, in the current study, morphine injection immediately preceded a (few seconds apart) the acetic acid administration in mice. A distinct experimental condition like the one used in the current study, and other characteristic behavioral sex differences noted in the latency to onset/peak occurrence of acute nociceptive behavior might have contributed to the disparities observed in the magnitude and direction of sex difference ($F > M$). For example, the co-administration of morphine and acetic acid might have offset the time-points between the latency to acetic acid-induced stretching responses (between 3 and 20 min) and morphine-induced antinociception (typically between 15-30 min via the IP route) in males, but not in females where delayed onset/occurrence of stretching (10-30 min) was time-dependently and effectively reversed by morphine (at the smallest dose tested). These results suggest that time-dependency may be an important variable both with respect to the onset and occurrence of peak nociception and antinociceptive effects of opioids in any given pain model, which could further interact with sex, species, and strain.

To our knowledge, there are no previous reports on the antinociceptive effects of oxycodone in the acetic acid-induced acute visceral nociceptive test (but see (203) for results with p-phenylquinone chemical assay). However, other studies, including one from our own laboratory, have established the antinociceptive effects of oxycodone using other acute thermal pain tests in rodents (200-205). While Beardsley and colleagues found equivalent potency for morphine and oxycodone in the acute chemical test reported only in male ICR mice (203), the present study results revealed greater potency for oxycodone (3.2-fold) over morphine in male C57Bl/6 mice, and equivalent efficacy in

both sexes. Several differences separate these two studies, including differences of mouse strains, chemicals used to induce nociception, and the addition of females in the current study. In comparison to morphine, while oxycodone produced greater antinociceptive effects in males, the opposite was observed in female mice (morphine 3.2 fold more effective than oxycodone) in the present study. The present results in male mice are consistent with previous reports that have demonstrated increased antinociceptive potency of oxycodone over morphine when administered systemically (intraperitoneal or subcutaneous) in male rats (162, 200, 201). It is well-established that both peripheral and central opioid receptors (specifically μ and κ receptors) mediate the antinociceptive effects of opioids in the acetic acid chemical test (206, 207). An interesting difference noted between morphine and oxycodone is that oxycodone has reduced μ -receptor binding potential (208) and reduced potency (> 10-fold difference) and efficacy than morphine in the GTP γ S-binding assay (209). Some researchers suggest that oxycodone's antinociceptive effects are mediated at least in part by κ receptors (154, 155). The differences in the antinociceptive potency between the two opioids might be mediated by different receptor populations at which they act (at least in part) or other pharmacokinetic factors (such as faster brain passage of oxycodone compared to morphine – see discussion (210)). Further, considering that other studies comparing the two opioids have reported increased potency for oxycodone over morphine in female rats (201, 210), the converse effect seen in female mice in the present study may be attributed to differences in species and pain models used across studies or perhaps route of administration.

With respect to the direction of sexual dimorphism in the sensitivities of rodents to oxycodone-induced antinociceptive effects, the results from previous studies using acute thermal pain tests have been mixed; either an increased effect in females (204) or no difference noted between sexes (201). The present study results are similar to the former one, in that oxycodone was more potent in the females compared to males (similar to the direction in sex difference observed for morphine in the present study), and extending these findings to mice using an alternative acute chemical pain test. Previous studies have investigated the potential role of pharmacokinetic factors in the sex differences in morphine antinociception and analgesia in rodents and humans. In humans, no significant differences in the plasma concentrations of morphine or its active metabolite (morphine-6-glucurodine) were observed between males and females following i.v. administration of morphine (211, 212). Similarly, the majority of studies in rats have demonstrated no sex-linked differences in the bioavailability of morphine, peak plasma or brain levels of the drug, and no differences in the elimination half-life or rate of decrease of brain concentrations of morphine following systemic subcutaneous administration suggesting that the sex differences may be less likely mediated by these pharmacokinetic factors (e.g. lipophilicity of the opioid) (187, 213). However, differential morphine metabolism between male and female rats (e.g. increased morphine-3-glucuronide to morphine-6-glucuronide ratios in females than males) has been previously attributed to the sex differences in morphine antinociception (214).

In addition to possible role of pharmacokinetic factors, the sex differences also appear to be linked to the inherent variations in the sensitivities of rodents to the effects of morphine as studies have shown sexual dimorphism in the inherent pain-related neural

system and responses to opioids (215) and the modulation of that system by sex hormones (165). In contrast, the mechanism(s) underlying sex differences for oxycodone's antinociceptive effects are largely unknown and very few studies have investigated the role of pharmacokinetic factors. For example, one study demonstrated increased systemic exposure of female rats to oxycodone, but increased systemic exposure of male rats to the inactive metabolite noroxycodone (216). This may support more antinociceptive effectiveness of oxycodone in females than in males as noted previously in rats (204) and in mice in the present study.

The novel and significant aspects of the current study using the chronic paclitaxel-induced peripheral neuropathy model are: i) a systematic assessment of paclitaxel-induced allodynia in an inbred genetically viable mouse strain; ii) the establishment of sex differences in sensitivities to the allodynic behavior in C67Bl/6 mice; iii) a characterization of the effects of Cremophor vehicle on allodynic behaviors in male and female mice. We have previously demonstrated that paclitaxel produced significant cold allodynia (1, 2, 4 and 8 mg/kg), but lacks clear dose-dependency in female C57Bl/6 mice (147), the results that are in agreement with a previous study comparing different mouse strains (217). The lack of effect for paclitaxel to induce cold allodynia in our previous study using lower doses of paclitaxel (1 and 2 mg/kg) in male mice, were extended using the higher doses of paclitaxel (4 and 8 mg/kg) in the current study. While the absence of paclitaxel-induced cold allodynia in male mice is inconsistent with reports in the literature, several differences including variability in paclitaxel dose and dosing regimens, behavioral testing, and mouse strains may account for such discrepancies between studies (217-219).

In contrast to paclitaxel-induced cold allodynia, the data revealed significant and increased sensitivity to paclitaxel-induced mechanical allodynia in both female and male C57Bl/6 mice. In comparison to the allodynic effects of 4 mg/kg dose of paclitaxel, the effects of 8 mg/kg dose (cumulative dose of 32 mg/kg) were more pronounced and persistent for at least 6-8 weeks after the first injection of paclitaxel in both sexes. Although the majority of the previous studies using a similar dose and dosing regimen of paclitaxel in mice have been in male subjects, the present study has extended these findings to female mice and the findings are consistent with the other studies in regard to the magnitude and time course of peak allodynia effects (between days 8 and 20) (220, 221). A recent study in rats focusing on sex differences had also demonstrated a similar and comparable mechanical allodynia time course in both sexes, although a lower dose of 2 mg/kg X 4 injections was used (191). These results in rats were essentially identical to the present findings in C57Bl/6 mice. With the mechanisms underlying paclitaxel-induced cold and mechanical allodynic behaviors still largely unknown (145, 188, 219), it is difficult to ascertain sex-specific factors that may support the selective sex differences observed in the paclitaxel-induced cold but not mechanical allodynic behaviors in mice seen in the present study. The present data also revealed that compared to baseline behavior, treatment with the Cremophor vehicle alone increased sensitivity of mice to cold (significant) and mechanical (trend in effect) stimuli. It is known that Cremophor produces several adverse effects in chemotherapy patients (222), and these data support the importance of testing the relative contribution of Cremophor to paclitaxel-induced painful peripheral neuropathy in humans.

Based on the above findings, a model of paclitaxel (8 mg/kg dose)-induced mechanical allodynia and saline control treatment replacing Cremophor (to minimize confounds from Cremophor vehicle-induced hypersensitivity) was incorporated to evaluate morphine's anti-allodynic effects in male and female mice. Systemic administration of morphine using a three-day dosing regimen resulted in dose-dependent increases in the paw withdrawal threshold in the paclitaxel-treated male and female mice, but with males revealing increased sensitivity to morphine's anti-allodynic effects (males: potency – 2.5 mg/kg) compared to females (potency – 5 mg/kg). These results support a larger preclinical literature demonstrating acute effects of morphine in the paclitaxel-induced model of neuropathy, and in other neuropathic/persistent pain models where similar sexual-dimorphism in morphine's effects ($M > F$) have been noted (191, 195, 221, 223-226). However, Hwang et al. (191) also used a similar paclitaxel-induced model of neuropathy and reported equivalent anti-allodynic effects of morphine at 2 and 5 mg/kg in male and female rats. While this is in contrast to our current findings in mice, it is possible that sex differences were not detected in their study because a lower dose of paclitaxel (2 mg/kg) was used to induce mechanical allodynia compared to a higher dose (8 mg/kg) used in the current study. This suggests a possible three-way interaction between the paclitaxel dose (magnitude of allodynic effects), sex, and morphine dose effects. Clearly more studies are needed to confirm these findings, to compare the consistency of these results both within and between species, and subsequently determine the mechanisms mediating sex-specific behavioral effects of opioids in the context of chronic persistent pain.

One limitation of the present study is that only adult gonadally intact male and female C57Bl/6 mice were tested using the two chemical-induced pain models, as opposed to also including gonadectomized adult mice. While sex hormones are one of the important factors influencing the difference in responses to pain and opioid antinociception/anti-allodynia between sexes (162), it is certainly a logical next step to delineate the potential underlying role of sex hormones in mediating the observed behavioral differences using these two chemical-induced models of pain in mice.

In summary, the present study demonstrates robust assay-specific sex differences in nociceptive and allodynic behaviors, and opioid-induced antinociceptive and anti-allodynic effects using two putative chemical-induced pain states in C57Bl/6 mice. In the acute acetic acid-induced nociceptive model, female mice were more sensitive to acute nociception and morphine's antinociceptive effects. Furthermore, this is the first documentation of oxycodone's effects in the acute acetic acid chemical assay in mice. While oxycodone's effectiveness was equivocal in both male and female mice, it was distinct from that of morphine, further being dependent on sex. Paclitaxel administration demonstrated enhanced sensitivity to non-noxious stimuli without affecting the overall health (no body weight or behavioral alterations, data not shown) of male and female mice. The present results suggest a lower threshold and an increased sensitivity to multiple stimulus modalities in females after paclitaxel treatment. Pronounced sex differences were observed in morphine's anti-allodynic effects with increased effectiveness observed in males. The comprehensive assessment of sexually dimorphic variations in the underlying nociceptive behaviors and opioid-induced antinociceptive/anti-allodynic behavioral effects using these two putative chemical pain

models provided a functional framework for the further characterization of the reward-related behavioral effects of opioids in the context of these pain-states in C57Bl/6 mice. These studies have been discussed in detail in the subsequent chapters 3 and 4.

CHAPTER 3

EFFECTS OF ACETIC ACID-INDUCED ACUTE NOXIOUS STATE ON THE DISCRIMINATIVE STIMULUS PROPERTIES AND CONDITIONED REWARDING EFFECTS OF PRESCRIPTION OPIOIDS IN MALE AND FEMALE MICE

Rationale

The majority of acute pain conditions are post-surgical and about 80% of the patients undergoing surgical procedures experience severe acute pain that is managed with prescription opioids as the primary choice of treatment in clinical settings (139). While it is a generally believed notion that addiction to prescription opioids is less likely to occur in acute pain settings (49), some reports highlight that even short term use of opioids for the treatment of moderate to severe acute pain requires caution due to the risk of diversion and addiction (30). In fact, a modest percentage of prescription opioid abusers have begun their prescription opioid misuse following legitimate prescriptions for pain from their physicians (227-229). The most commonly abused prescription opioid drugs include the semi-synthetic mu-agonist narcotics, including the derivatives of morphine and codeine (hydromorphone, oxycodone, and hydrocodone) (230, 231). These drugs display comparable abuse liability and reinforcing potencies (230, 232-234) and are first line treatments for severe acute pain in adults in the clinical settings (29, 30). This raises an important question of whether acute pain can modulate the subjective experience of opioids and alter the rewarding potential of these drugs.

Only a few studies in the recent past have started to systematically address this question. For example, experimentally-induced pain (cold-pressor test) in non-drug-

abusing humans has been demonstrated to attenuate the ratings of fentanyl-induced subjective effects of ‘feeling elated’ (euphoria) (109), and morphine-induced subjective effects including ‘coasting’, ‘high’, ‘lightheadedness’. In contrast, an alternative opioid, butorphanol increased ratings of ‘elated (very happy)’ (110). In contrary, for the prescription opioid oxycodone, empirical findings in humans reveal that positive subjective effects and abuse liability measures do not vary as a function of experimentally induced pain (73, 111). These results indicate that the subjective effects of prescription opioids may vary as a function of pain, but may be dependent on the type of prescription opioid being studied.

Since the literature encompassing sex differences in pain behaviors and opioid-induced analgesic effects are plentiful (explained and reviewed in chapter 2), recent studies have started to expand this area of research to establish sex differences in other behavioral effects of opioids, including the abuse-related behavioral effects of prescription opioids in animals and humans (156). Of particular interest are studies revealing sex differences in the abuse-related and subjective effects of prescription opioids in humans (62, 71, 72, 111). For example, in healthy volunteers, morphine and oxycodone were shown to produce increased positive subjective effects in males (62), but increased dysphoric and negative effects (e.g. nausea) in female (62, 71, 72). However, these sex-specific effects are also known to vary as a function of drug use status/history because in sporadic prescription opioid abusers, females were more sensitive to a number of abuse liability measures of oxycodone (street value, high, and drug liking) (111). Similarly, sex differences in the rewarding/reinforcing effects of the prescription opioid morphine have been assessed preclinically in rodents (greater effects in female rats

compared to males) (76, 77, 235), although little is known about other prescription opioids such as oxycodone. While gender-based studies in humans and animals have evaluated sex differences in opioid-induced reward, the role of gender in the modulating effects of pain on the abuse liability of prescription opioids has received little attention. To date, only two studies have been reported in humans: Zacny and Beckman (236) showed that cold pressor pain attenuated the subjective effects of butorphanol selectively in females, while Lofwall and colleagues (111) demonstrated that the abuse liability measures of oxycodone do not vary a function of pain in male and female opioid-abusers. However, the modulating effects of acute experimentally induced pain on the reward-related behavioral effects of prescription opioids as a function of sex/gender have not been examined preclinically.

The drug discrimination assay is a well-established procedure and a number of studies have demonstrated opioid discrimination, particularly morphine, in different animal species (237-240). Some studies in rats have shown significant sex differences in the discriminative stimulus effects of morphine with females demonstrating greater sensitivity to morphine-induced stimulus effects when compared to males (241-243). However, the stimulus properties of morphine in mice have only been studied using passive avoidance or simple two-compartment shuttle box procedures (244, 245), and to date, morphine or oxycodone drug discrimination using operant techniques has not been studied in mice. Conditioned place preference (CPP) is a commonly used behavioral procedure that is used to measure the rewarding effects of drugs (246). As similar as the discriminative and rewarding/reinforcing effects of opioids such as morphine appear to be, studies have attributed the two opioid-induced behaviors to different neuroanatomical

brain regions and mediated by different underlying processes, potentially by distinct brain regions (247).

The presents study: i) established the discriminative stimulus properties of morphine and oxycodone in male and female C57Bl/6 mice; ii) determined the modulating effect of the acute acetic acid-induced noxious state on the discriminative stimulus effects of morphine and oxycodone in male and female C57Bl/6 mice; iii) assessed the conditioned rewarding effects of the prescription opioid morphine in the presence of an acetic acid-induced acute noxious state in male and female mice using the conditioned place preference procedure.

Methods

Subjects

Male C57Bl/6 mice weighing 20-25 g, and female mice weighing 15-20 g, and all mice 5-6 weeks of age, were purchased from SAGE Labs, (Boyertown, PA, USA) for the drug discrimination studies (N=13, males; N=15, females) and from Taconic, Farms, Inc. (Cranbury, NJ, USA) for the conditioned place preference experiments (N=130, males; N=122, females). Mice were group housed in plastic cages and allowed to acclimate to the temperature-and humidity-controlled animal facility for 3 to 7 days before the experiments began. Mice were housed under a 12 h light/dark cycle with lights off at 10.00 h, so that all experiments occurred during the dark phase of the diurnal cycle. For the conditioned place preference studies, mice were housed in groups of four and food and water were available *ad libitum*. For the drug discrimination studies, mice were individually housed one day prior to commencement of the experiment and maintained at

90% of their free feeding body weights feeding approximately 2.75 g pellet daily of Purina Rodent Chow Diet 5001 (Ralston-Purina, St. Louis, MO, USA) in addition to the food earned in the experiment for the remainder of the studies. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University and the Guide for the Care and Use of Laboratory Animals (Institution of Laboratory Animal Research, National Academy Press; Eighth edition, revised 2011).

Drugs

Morphine sulfate and oxycodone hydrochloride were generously donated by the National Institute on Drug Abuse (NIDA) drug supply program (Bethesda, MD, USA). Morphine and oxycodone were dissolved in 0.9% saline. A stock solution of 1 N acetic acid was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and diluted to 0.4% concentration solution in sterile water. All injections were administered intraperitoneally (IP) in a volume of 0.01 ml/g of body weight.

Experiment 3: Opioid Drug Discrimination

Apparatus

Experiments were conducted in twelve chambers (21.6 cm x 17.8 cm x 12.7 cm, Model ENV-307W, Med Associates, Georgia, Vermont, USA) located within ventilated sound attenuating enclosures. One wall of the chamber contained two nose poke holes on the left and right each 1.2 cm in diameter with internal amber stimulus lights (ENV-313W) and two amber stimulus lights mounted on the wall directly above the two nose-

poke holes. A food receptacle, the center dipper hole was present between the two-nose pokes that opened to a motor-driven dipper (ENV-302W) for liquid food presentation. The opposite wall of the chamber contained a house light (ENV-315M), tone generator (ENV-323AW), and ventilator fan that generated white noise. The center receptacle for dipper access contained an amber stimulus light located above it (ENV-221M). All experimental contingencies were arranged and the data were recorded by a computer-driven interface (Model SG-503, MED Associated, St. Albans, VT, USA).

Procedure

Mice were trained to discriminate saline or morphine (3.2 mg/kg) using a two-choice, two-trial training procedure adapted and modified from Walker and Young (237). Details of the training and test phases of the assay are outlined below:

Training phase. During acquisition of operant responding for 50% liquid Ensure solution, male and female mice were shaped to nose-poke in the right nose-poke initially under an FR-1 schedule of reinforcement. Following stable responding by each mouse for 3 consecutive days as defined as at least 50 responses in the right nose-poke and less than 10% of total responses in the left nose-poke, saline and 3.2 mg/kg morphine were introduced as discriminative stimuli for food-reinforced responses using a two-trial training procedure. Each training session included two discrete trials, each consisting of a 15-min pretreatment period followed by a 5-min ratio component. Before each trial, mice received an injection of saline or morphine, and then were placed in the darkened experimental chambers. After 15 min, stimulus lights were illuminated and liquid food was delivered initially under FR-1 schedule that was gradually increased to an FR-10

schedule of reinforcement over consecutive training sessions. During training sessions, left nose-poke responses were reinforced after an injection of morphine and right nose-poke responses were reinforced after an injection of saline. During any given discrete trial session, incorrect nose-poke responses reset the ratio counter to 0. The ratio component terminated after 10 reinforcers or 5 min, whichever occurred first. After the first 5-min component, mice were removed from the chamber, injected with saline or morphine, and returned to the chamber for their second trial. Saline trials in the first component were followed by an additional saline trial or a morphine trial. A morphine trial in the first component was followed by a second trial with nose-pokes reinforced in the morphine nose-poke, although this second trial was preceded by a saline injection to control for handling and the injection procedure. Training sessions occurred 5-7 days per week. All mice had to attain the following criteria on seven consecutive trials before the first test session: 1) fewer than 10 responses on the incorrect nose-poke before the first reinforcer, and 2) greater than 80% injection-appropriate responding over the entire session. After initial testing criteria were met, test sessions were interspersed with at least two training sessions and if a mouse failed to meet criteria, then the mouse received at least 1 week of accurate training sessions.

Test phase. During two-trial testing sessions, saline or a dose of morphine (0.1-10 mg/kg), or two doses of morphine were administered, where saline or a dose of morphine was administered before the 15-min pretreatment period, and 10 consecutive responses in either nose-poke produced liquid food reinforcement in a 5-min ratio component. After completion of the first 5-min ratio component, mice were removed, injected with a

dose of morphine following saline test or the next cumulative dose of morphine (0.25, 0.5 or 1.0 log₁₀-unit increments) following the morphine test, and placed back into the chamber for the second pretreatment period. After a morphine discrimination dose-response effect was completed, the dose-response effect for oxycodone (0.1-3.2 mg/kg) was generated by substitution tests. Doses of oxycodone were tested in a similar manner as morphine either alone or cumulatively in the two discrete sessions (0.5 or 1.0 log₁₀-unit increments).

Effect of acute acetic acid-induced noxious state on opioid discrimination. After the dose-response curves for morphine and oxycodone were completed, discriminative effects were re-determined in the presence of acute acetic acid-induced noxious state. During the two-trial testing sessions, saline, morphine or oxycodone were administered along with 0.4% acetic acid (one injection on each side of the peritoneal cavity) before the 15-min pretreatment period in the experimental chambers and 10 consecutive responses in either nose-poke produced liquid food reinforcement in a 5-min ratio component. After the completion of the first ratio component, the second pretreatment time was began without a second injection, and the second ratio component followed. Data recorded from the first trial were excluded from further analyses for two reasons: (i) significant rate suppressing effects of acetic acid/drug combinations were observed in the first trial for both female and male mice (Figure 8, panels A and B); ii) mice were engaged in increased stretching behavior during the first ratio component (15 to 20 min following acetic acid administration) compared to the second ratio component (35 to 40 min following acetic acid administration) (Figure 9).

To minimize possible confounds in determining the selective effects of acetic acid-induced noxious state on the opioid-discriminative effects, only data from the second discrete-trial time point, when the rates of operant responding were similar to baseline conditions (Trial 2 bars: Figure 8, panels A and B) and the number of stretches (sensory reflexive response to the acetic acid stimulus) were significantly attenuated (Figure 9, black bars), were used to analyze the modulatory effect of the acute noxious pain-state on opioid-induced discriminative cues in mice. Tests with acetic acid were performed with at least 5 to 7 days between tests to avoid acetic acid-induced peritonitis or damage to the viscera in mice, as noted previously in our laboratory (Neelakantan et al., under review).

Mice showing any changes in the discriminative stimulus effects of morphine or oxycodone in the presence of the acetic acid-induced noxious state, mice were further tested with the corresponding opioid and sterile water (vehicle condition for acetic acid) combination for three discrete trials to confirm the following: i) that the effects of the drug and acetic acid combinations were selective effects and not due to non-selective behavioral effects that the combinations might otherwise produce (e.g. handling of mice, two simultaneous injections, or stress); and ii) that the discriminative stimulus effects of the drug were persistent at least until the second discrete trial time point (time point at which data were collected (i.e. 35 min post drug administration) and used for the analyses).

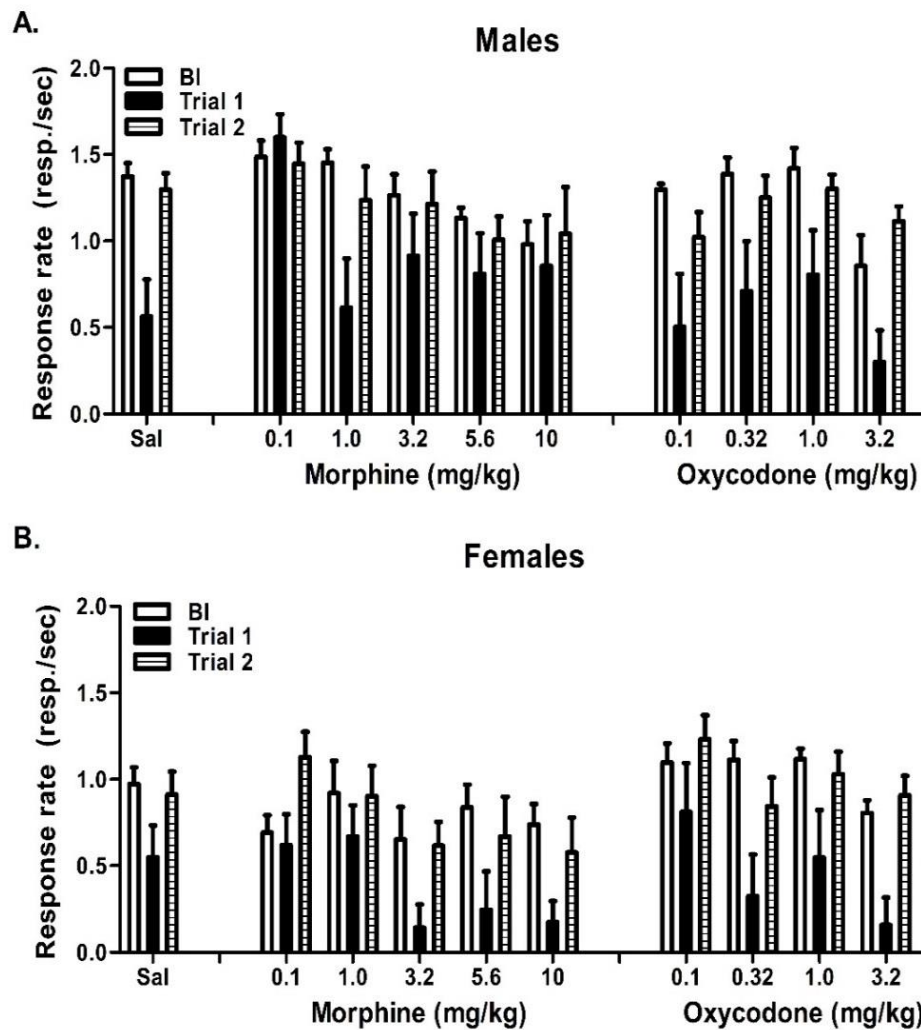


Figure 8. Effects of saline, morphine or oxycodone alone (baseline, BI) and in combination with acetic acid (0.4 %) (trials 1 and 2) on the response rates as determined from drug discrimination discrete trial sessions. Abscissa: Dose of drugs in mg/kg or saline (0.1 ml/kg) [Sal: Saline control condition] administered alone or in combination with acetic acid (trials 1 and 2). Ordinate: Response rate, responses (resp) elicited per unit time (resp/sec). Each bar represents the mean number of responses per unit time (+SEM) achieved within each session at baseline or drug alone condition (BI, clear bar), drug+acetic acid conditions in trial 1 (filled bar) and trial 2 (pattern bar);. Two-way ANOVA revealed significant main effect of dose and trial as factors but not an interaction in both male (panel A, n = 5-7) and female (panel B, n = 5-11) mice. Trial: males- [$F_{(2, 144)} = 21.49, p < 0.0001$], females- [$F_{(2, 164)} = 23.05, p < 0.0001$]; Dose: males- [$F_{(9, 144)} = 3.42, p = 0.0008$], females- [$F_{(9, 164)} = 3.29, p = 0.001$].

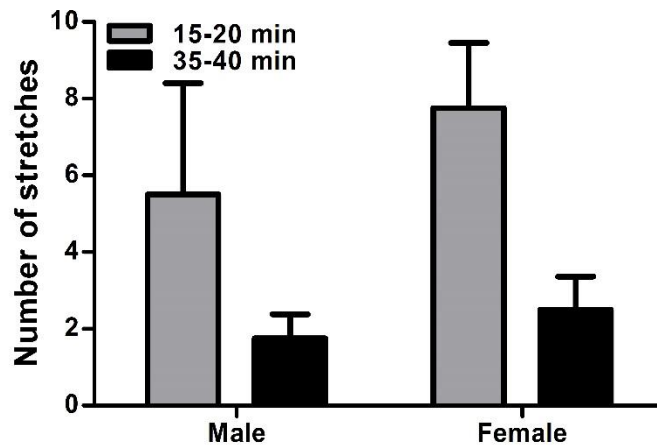


Figure 9. Time course of the sensory reflexive visceral stretching responses induced by acetic acid in male and female C57Bl/6 mice. Abscissa: sex of mice. Ordinate: number of stretches recorded and measured during two 5-min bins: 15- to 20-min (grey bars) and 35- to 40-min (black bars) post-administration of saline+acetic acid (0.4%) on either side of the peritoneal cavity. Each bar represents the mean number of stretches in the two 5-min bins (+SEM) (n = 4/ group). Two-way ANOVA revealed a significant main effect of time as factor [$F_{(1, 12)} = 6.5, p = 0.0255$].

Data and Statistical Analysis

Dose-response curves for the discriminative stimulus effects of morphine and oxycodone and the secondary measure of response rates were analyzed using repeated measures one-way ANOVA to confirm dose-dependency in the data for male and female mice. ED_{50} values for the discriminative effects of morphine and oxycodone were determined for male and female mice from the linear portion of the dose-response curves using linear regression analysis (GraphPad Prism 5.0 Software, Inc, La Jolla, CA). To determine the effects of acetic acid on the discriminative stimulus effects of opioids in mice, individual ED_{50} values were calculated for every mouse that completed the dose-response curve for the opioid+acetic acid testing condition. Subsequently, individual

ED₅₀ values were averaged into group means and compared to their respective baseline (drug alone) ED₅₀ values using paired Student's t-test. Two-way ANOVAs were used where necessary to determine the significant effects of factors such as morphine dose and discrete trials or treatment (control vs acetic acid) and morphine dose, and the interaction between these factors within each sex. Statistical significance was set at P<0.05 for all analyses.

Experiment 4: Morphine Conditioned Place Preference (CPP)

Apparatus

The CPP apparatus consisted of 8 identical experimental chambers (Model ENV-3013, MED Associates, St. Albans, VT, USA), each with white (white walls and mesh flooring), middle grey, and black (black walls and bar flooring) compartments containing distinct cues and light settings. Each chamber was located within a sound-attenuating enclosure and connected to a computer-driven interface (Model SG-6080/D, MED Associates, St. Albans, VT, USA) that controlled the data collection and experimental conditions.

Procedure

Conditioned place preference assay. The CPP assay is highly subject to procedural manipulations. Therefore, prior to beginning experiments, pilot studies were performed to define the parameters of the CPP assay in a manner that produced robust morphine-induced place preference in C57Bl/6 mice. Based on a biased design concept reported by Bardo and colleagues (246), we conducted parametric studies in our

laboratory to determine if a rewarding drug such as morphine has to be paired with the preferred or the non-preferred side of a biased CPP apparatus. The mice revealed a significant white side bias (see Figure 10) in the CPP apparatus without any drug manipulations. This is represented by a significant aversion to the black side (Figure 10, panel A: control bar) or greater than a 200 s preference for the white side (Figure 10, panel B: control bar) seen in C57Bl/6 mice that were conditioned with vehicle injections on both sides of the CPP compartments. Further, when morphine (10 mg/kg) was paired with the non-preferred black side, it only removed the aversion to a certain degree (Figure 10, panel A: Mor bar), while when morphine was paired in the preferred white side it produced a significant increase in the time spent in the white side in mice over the preference of saline-paired mice demonstrating a significant conditioned place preference (Figure 10, panel B: Mor bar). This modified procedure, *pseudo-biased place-conditioning procedure*, where the white side of the CPP compartment (typically preferred side) is paired with the drug was employed for the subsequent experiments. Respective control groups, in which mice were conditioned with vehicle injections on both sides of the CPP compartments were included in all experiments and the comparisons for the drug-paired groups were made against the control groups making this a between group design.

Morphine-induced CPP in male and female mice. Male C57Bl/6 mice (n=48) and, female C57Bl/6 mice (n=51) were divided into six groups (n=6-11/group): saline/saline group, saline/morphine (0.3-10 mg/kg) groups. On Day 1 of conditioning, all mice received saline intraperitoneal (IP) injections and were placed in the black compartment

of the CPP apparatus following a 15-min pretreatment time and conditioned for a period of 30 min. On Day 2, control mice received saline while the treatment groups received one of the doses of morphine (0.3-10 mg/kg) and placed in the white compartment following a 15-min pretreatment time and conditioned for a period of 30 min. Similar pairings were repeated until Day 6. On Day 7, mice were given free access to all the chambers for 30 min following an initial 5-min acclimation in the middle grey compartment. Time spent in the white, black, and middle grey compartments in a drug-free state were recorded on the test day and the preference score was calculated for each mouse.

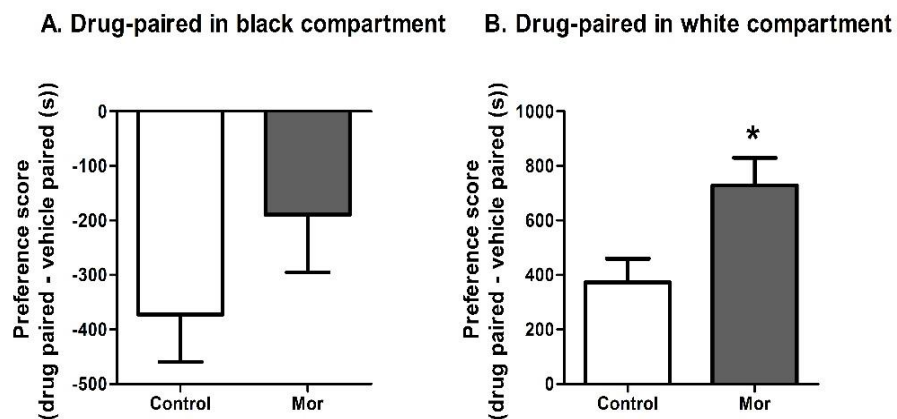


Figure 10. Effects of morphine (10 mg/kg) using a pseudo-biased CPP procedure. Morphine is paired in the black (panel A) and white (panel B) compartments of the CPP apparatus. Abscissa: Treatment groups: Control received vehicle (saline) injections in both compartments; Mor received morphine (10 mg/kg) injections every other day in the black (A) or white compartment (B). Ordinate: Preference score (s): time spent in the drug - vehicle paired sides on the test day. Each bar represents mean preference score \pm SEM (n = 8/ group). *, p < 0.05 compared to control as determined by an unpaired t-test.

Effect of acetic acid-induced noxious state on the conditioned rewarding effects of morphine. Male (n = 69) and female (n = 71) C57Bl/6 mice were divided into control

and treatment groups. The same procedure outlined in the above section was followed. On the conditioning days, control mice received intraperitoneal injections of sterile water 10 min before (placed in their home cage during pretreatment) saline injections (15 min pretreatment in home cage) and conditioned alternating between the two compartments of the CPP apparatus for 6 days. The other treatment group received intraperitoneal injections of sterile water 10 min before (placed in their home cage) saline injections on the saline conditioning days (days 1, 3, and 5) and acetic acid (0.4% concentration) injections 10 min before (placed in their home cage) morphine injections on the drug conditioning days (days 2, 4, and 6). Acetic acid injections administered prior to morphine administration on the morphine conditioning days in the treatment group of mice produced a brief acute noxious state characterized by acid-induced stretching behavior such as body contortions and extension of the hind limbs. Three morphine doses were chosen to be tested in this experiment based on the dose response effect for morphine-induced place-preference (results of the previous experiment) as determined in male and female mice: a low non-rewarding dose (0.3 mg/kg), an intermediate rewarding dose (2.5 mg/kg), and a high rewarding dose (10 mg/kg) of morphine. The effect of the acute noxious state on morphine-induced reward was determined by comparing the preference for the morphine-paired compartment (dose-response effect for morphine) between the control and treatment groups.

Data and Statistical Analysis

Time spent in each compartment in a drug-free state was recorded on the test day and used to calculate the preference score for each mouse using the following equation:

$$\text{Preference score} = [\text{Time spent in the morphine-paired compartment}] - [\text{Time spent in the saline-paired compartment}]$$

Separate one-way ANOVAs (GraphPad Prism 5.0 Software, Inc, La Jolla, CA) with Tukey multiple comparison posthoc tests were used to determine the sensitivities to morphine-induced conditioned rewarding effects within naïve, control, and acetic acid treated male and female groups of mice. To determine the interactions between sex and dose and the factors treatment and dose within each sex, two-way ANOVAs were used. Statistical significance was set at $p < 0.05$ for all analyses.

Results

Experiment 3: Opioid Drug Discrimination

Morphine was effectively established as a discriminative cue in both male and female C57Bl/6 mice using the two-choice operant drug discrimination procedure. Morphine produced dose-dependent stimulus effects (males and females: $p < 0.0001$) and rate-decreasing effects (males: $p = 0.0002$; females: $p = 0.0029$) in both male (Figure 11, panels A and B) and female mice (Figure 11, panels B and D). The discriminative stimulus effects of morphine were equivalent in potency and efficacy in both male and female C57Bl/6 mice (ED_{50} [C.L.] = 0.67 mg/kg [0.19-2.2] (female); ED_{50} [C.L.] = 0.5 mg/kg [0.1-2.0] (male)). Oxycodone dose-dependently substituted for morphine in both male ($p = 0.0062$) and female ($p = 0.0001$) mice and produced significant dose-dependent

rate-decreasing effects (males: $p = 0.0294$; females: $p = 0.0027$). Relative to morphine, oxycodone was found to be marginally more potent (1.5-fold) in male mice (ED_{50} [C.L.] = 0.28 mg/kg [0.024-1.7]) while no potency differences were observed between morphine and oxycodone (ED_{50} [C.L.] = 0.68 mg/kg [0.23-2.2]) in female mice.

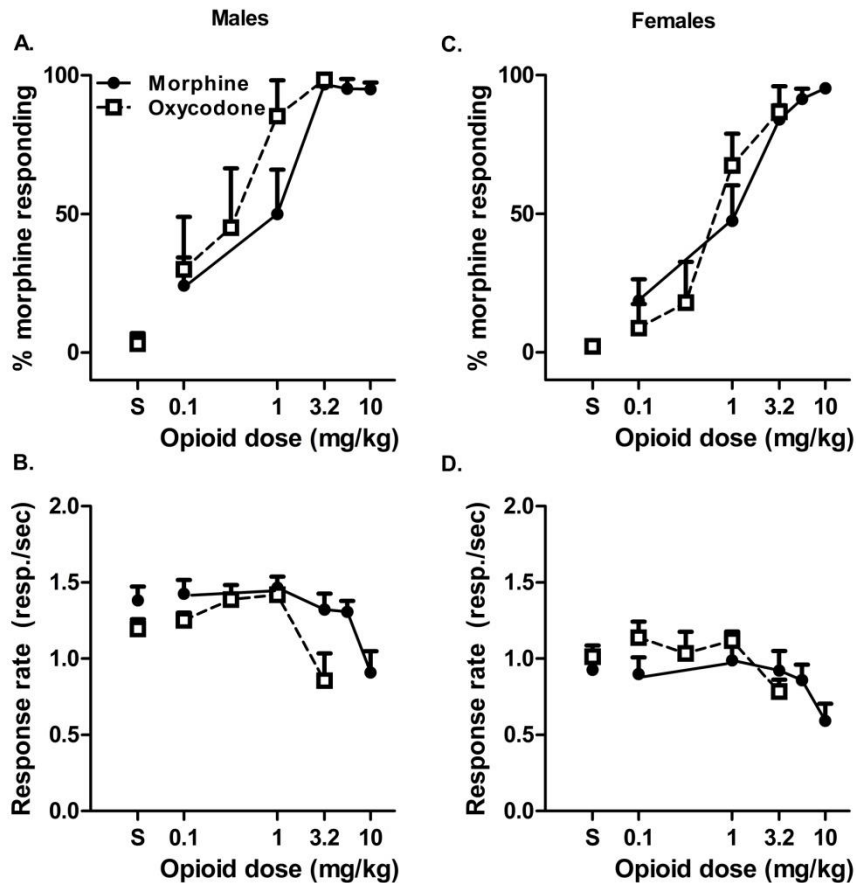


Figure 11. Discriminative stimulus and rate-decreasing effects of morphine (filled circles and solid line) and oxycodone (open squares and dotted lines) in male (panels A and B, $n = 5-9$) and female (panels C and D, $n = 5-10$) C57Bl/6 mice. Abscissa: Dose of the opioid expressed as mg/kg. Ordinate: % of total responses directed to the training drug (morphine)-appropriate nose poke (top panels A and C); Responses per unit time (resp./sec) (bottom panels B and D). S: saline. All points represent the mean % of morphine-appropriate responding (panels A and C) or mean response rates (panels B and D) in the respective groups + SEM.

Acetic acid-induced acute noxious state selectively attenuated the sensitivity of male mice to the discriminative stimulus effects of morphine but not oxycodone (Figure 12, panels A and C). A significant rightward shift in the dose-response curve for morphine was observed without any changes in the efficacy of morphine's discriminative effects (no downward shift in the dose-response curve) when morphine was tested in combination with acetic acid in male mice (Figure 12, panel A). A significant 2.2-fold decrease in potency ($p < 0.05$) was revealed by paired t-test analyses of the ED_{50} values for the discriminative stimulus effects of morphine alone and in combination with acetic acid for mice that had tested under both dose-response conditions (see Table 3). No significant differences were observed between the rate-decreasing effects of morphine alone or morphine when administered in combination with acetic acid (Figure 12, panel B). Further, two-way ANOVA revealed significant main effects of acetic acid treatment [$F_{(1,30)} = 12.09$, $p = 0.0016$] and morphine dose [$F_{(2,30)} = 7.36$, $p = 0.0025$], but no significant interaction was observed [$F < 1.0$] (Figure 13, panel A). For the time-dependent effects of morphine's discriminative cues in male mice, a two-way ANOVA revealed a significant main effect of dose [$F_{(2,39)} = 3.36$, $p = 0.0451$], but no effect of discrete trials/time points or an interaction between time and dose [$F < 1.0$] (Figure 13, panel B). In contrast to the modulatory effects of acetic acid on the discriminative stimulus effects of morphine, acetic acid-induced noxious state neither altered the potency nor efficacy of the oxycodone substitution (Figure 12, panel C; Table 3), nor oxycodone-induced rate-decreasing effects in morphine-trained male mice (Figure 12, panel D).

Males

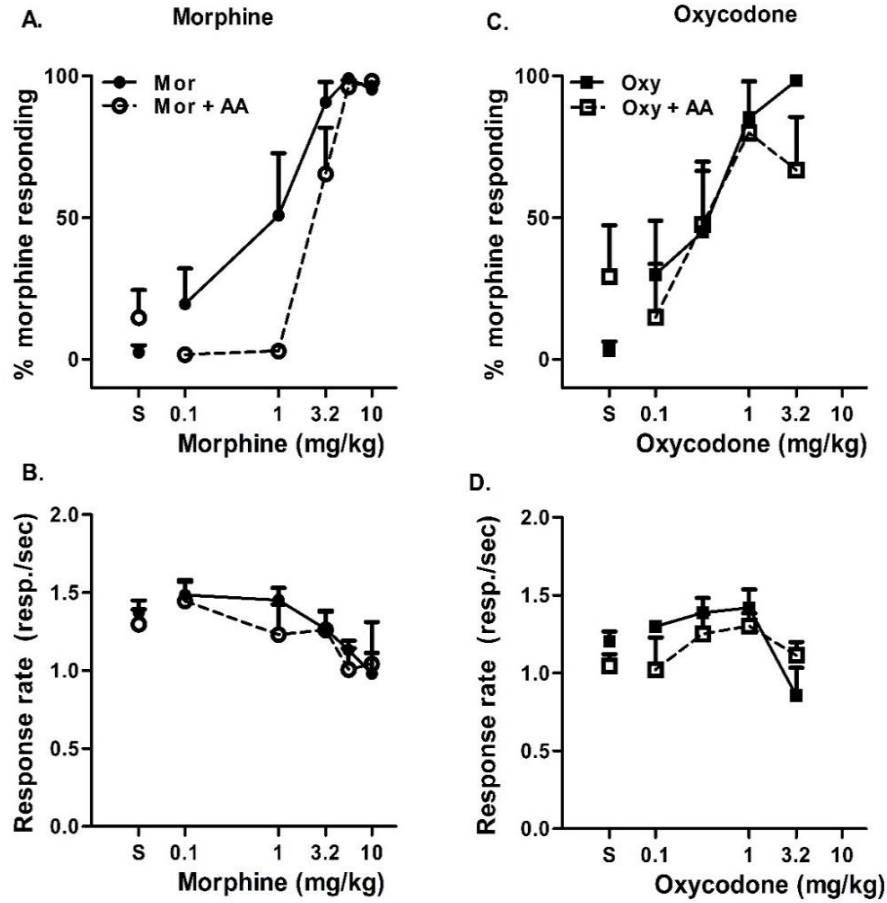


Figure 12. Effects of acute acetic acid-induced noxious state on the discriminative stimulus effects of morphine (panels A and B) and oxycodone (panels C and D) in male mice. Abscissa: Dose of the opioid, morphine (panels A and B) and oxycodone (panels C and D) expressed as mg/kg. Ordinate: % of total responses directed to the morphine-appropriate nose poke (panels A and C); Rate of responding (resp./sec) (panels B and D). S: saline; Mor: morphine alone (filled circles, solid line); Mor+AA: morphine+acetic acid (0.4%) (open circle, dotted line); Oxy: oxycodone alone (filled squares, solid line); Oxy+AA: oxycodone+acetic acid (0.4%) (open squares, dotted line). Each point represents the mean % morphine-appropriate responding (panels A and C) or mean response rates (panels B and D) in $n = 5-7$ mice/group + SEM.

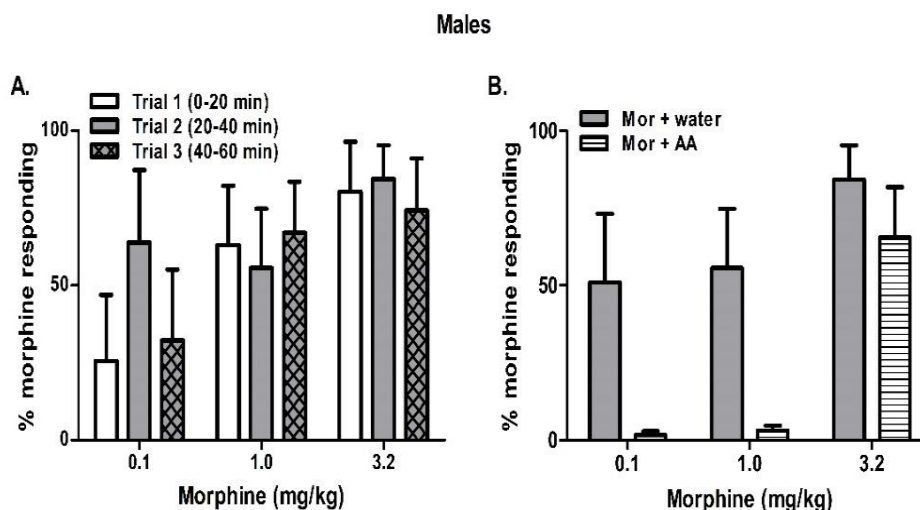


Figure 13. Time course of morphine-induced discriminative effects (panel A) and effects of acute acetic acid-induced noxious state compared to vehicle administration (water) on the discriminative stimulus effects of morphine (panel B) in male mice. Abscissa: Dose of morphine expressed as mg/kg. Ordinate: % of total responses directed to the morphine-appropriate nose poke; Bars represent the mean % morphine-appropriate responding during the three discrete trials after administration of water+respective morphine doses [panel A]: trial 1 (clear bars), trial 2 (grey bars), trial 3 (pattern bars); [panel B]: mean % morphine-appropriate responding recorded from trial 2 after administration of morphine+water (Mor+water, grey bars) *versus* morphine+0.4% acetic acid (Mor+AA, pattern bars) + SEM (n = 4-6/ group).

Table 3. Summary of statistical analyses for the effects of acetic acid-induced noxious state on the discriminative stimulus effects of morphine and oxycodone in male and female mice.

Treatment	Males		Females	
	Morphine ED ₅₀ values ±SEM mg/kg (n = 5)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 5)	Morphine ED ₅₀ values ±SEM mg/kg (n = 7)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 5)
Drug alone	0.91 ± 0.37	0.48 ± 0.19	0.76 ± 0.23	0.82 ± 0.22
Drug + Acetic acid (0.4%)	2.03 ± 0.51*	0.36 ± 0.085	1.5 ± 0.44	1.07 ± 0.21

*, p < 0.05

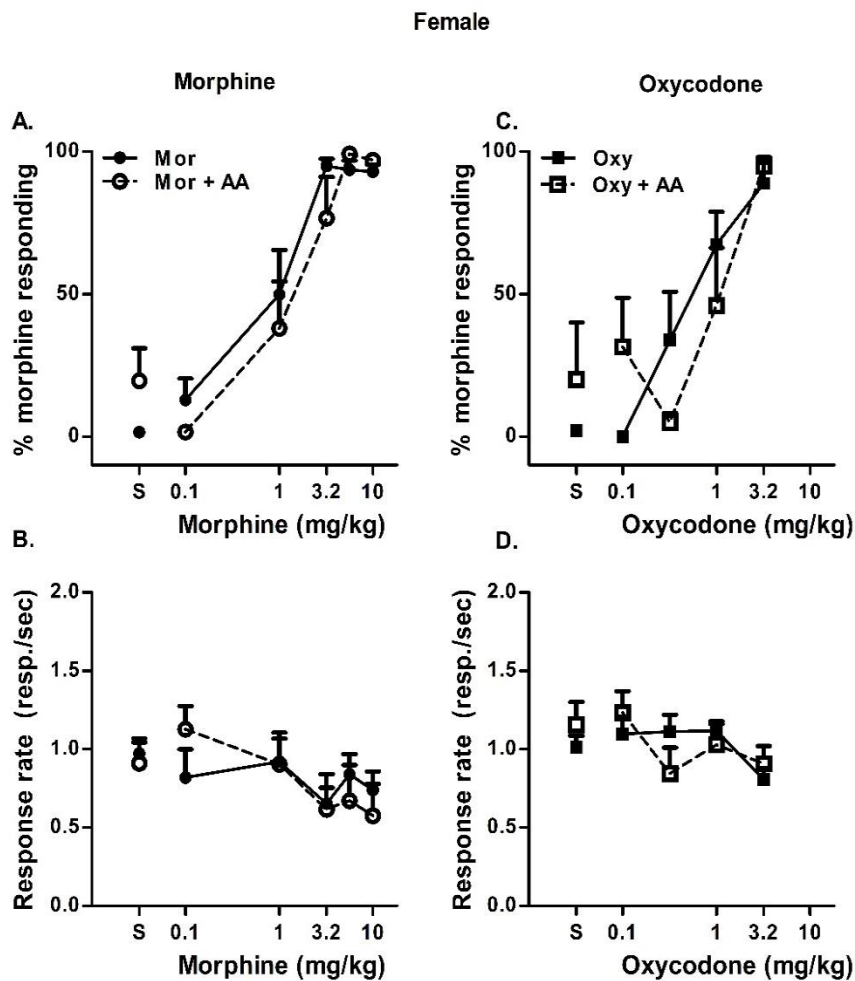


Figure 14. Effects of acute acetic acid-induced noxious state on the discriminative stimulus effects of morphine (panels A and B) and oxycodone (panels C and D) in female mice. Abscissa: Dose of the opioid, morphine (panels A and B) and oxycodone (panels C and D) expressed as mg/kg. Ordinate: % of total responses directed to the morphine-appropriate nose poke (panels A and C); Rate of responding (resp./sec) (panels B and D). S: saline; Mor: morphine alone (filled circles, solid line); Mor+AA: morphine+acetic acid (0.4%) (open circle, dotted line); Oxy: oxycodone alone (filled squares, solid line); Oxy+AA: oxycodone+acetic acid (0.4%) (open squares, dotted line). Each point represents mean % morphine-appropriate responding (panels A and C) or mean response rates (panels B and D) in $n = 5-7/$ group + SEM.

Acetic acid-induced acute noxious state failed to alter the discriminative stimulus effects of morphine in female mice (Figure 14, panel A). Paired t-test analyses of the ED₅₀ values for the discriminative stimulus effects of morphine *versus* morphine+acetic acid revealed a subtle change in potency that was not statistically significant (see Table 3). The rate-decreasing effects of morphine were not further altered by acetic acid (Figure 14, panel B). Similarly, acetic acid-induced noxious state neither altered the potency/efficacy of the oxycodone substitution (Figure 14, panel C; Table 3), nor the oxycodone-induced rate-decreasing effects in the morphine-trained female mice (Figure 14, panel D).

Experiment 4: Morphine Conditioned Place Preference (CPP)

Morphine produced conditioned rewarding effects in male and female C57Bl/6 mice, however differences were observed between sexes. The conditioned rewarding effects of morphine were found to be dose-dependent in male mice ($p < 0.0001$) with the higher doses of morphine (5 and 10 mg/kg) producing significant rewarding effects compared to the control (saline) ($p < 0.0001$) and the lowest dose of morphine tested (0.3 mg/kg, $p < 0.0001$) (Figure 15). In contrast, female mice acquired morphine CPP at a lower dose (1 mg/kg), but the conditioned rewarding effects of morphine were relatively dose-independent compared to males (Figure 15). A two-way ANOVA revealed significant main effects of sex [$F_{(1, 92)} = 12.49$, $p = 0.0006$], dose [$F_{(5, 92)} = 108.24$, $p < 0.0001$], and a significant interaction [$F_{(5, 92)} = 18.09$, $p < 0.0001$] between the factors. Although the magnitude of morphine reward at the higher doses (5 and 10 mg/kg) appeared to be greater in male compared to female mice, the posthoc tests did not reveal statistical significances.

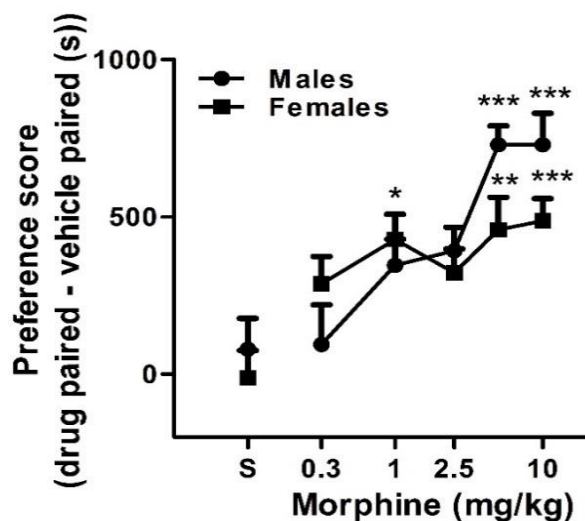


Figure 15. Conditioned rewarding effects of morphine in male (filled circles) and female (filled squares) C57Bl/6 mice. Abscissa: Control (S: saline), or doses of morphine expressed as mg/kg. Ordinate: Preference score (s): time spent in the drug - vehicle paired sides on the test day. Each point represents the mean preference score + SEM (n = 7-14/ group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ compared to the respective saline (S) groups within each sex as determined by a one-way ANOVA.

Acute acetic acid-induced noxious state did not alter the sensitivities of male and female mice to the conditioned rewarding effects of morphine (Figure 16, panels A and B). In the control male group, morphine produced dose-dependent rewarding effects ($p = 0.0026$) and the posthoc tests revealed significant differences in morphine reward at 10 mg/kg dose compared to saline ($p < 0.05$) and 0.3 mg/kg dose ($p < 0.01$). In the control female group, morphine displayed a trend toward dose-independent rewarding effects (Figure 16, panels A and B) with the data not reaching statistical significance due to increased variability in the saline-conditioned group. These results for the control male and female groups were similar to the conditioned rewarding effects of morphine observed in naïve groups of male and female mice (results of the previous experiment, Figure 15).

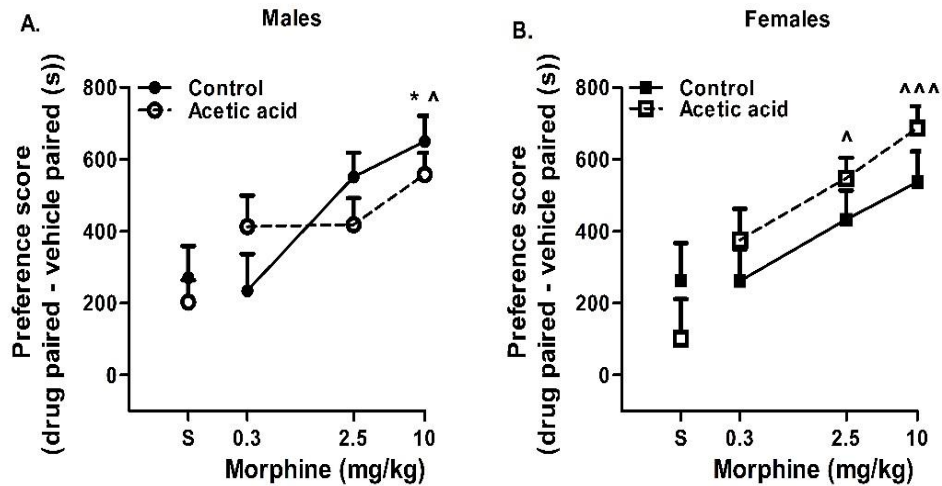


Figure 16. Effects of acetic acid-induced noxious state on the conditioned rewarding effects of morphine in male (panel A) and female (panel B) mice. Abscissa: Control (S: saline), or doses of morphine expressed as mg/kg. Ordinate: Preference score (s): time spent in the drug - vehicle paired sides on the test day. Each point represents the mean preference score + SEM (n = 7-15/ group). *, p < 0.05: compared to saline (S) in the control group within each sex. ^, p < 0.05; ^^^, p < 0.001 compared to saline (S) in the acetic acid treatment group within each sex as determined by a one-way ANOVA.

Acetic acid treatment showed a trend toward an increase in sensitivity to the conditioned rewarding effects of morphine in female mice. A two-way ANOVA revealed a significant main effect of dose [$F_{(2, 49)} = 5.81, p = 0.0054$], a trend in the main effect of treatment [$F_{(1, 49)} = 3.2, p = 0.0799$], but no significant interaction [$F < 1.0$] in female mice. In contrast, acetic acid treatment produced no change in the sensitivities of male mice to the conditioned rewarding effects of morphine. A two-way ANOVA revealed a significant main effect of dose [$F_{(2, 48)} = 5.47, p = 0.0073$], but no effect of treatment or an interaction [$F < 1.0$].

Discussion

The preclinical assessment of the stimulus properties of prescription opioids as a function of pain and in both sexes is clinically relevant and this study is the first to our knowledge to empirically address this question. The current study examined the modulating effects of the presence of acute acetic acid-induced noxious state on the discriminative stimulus properties of prescription opioids (morphine and oxycodone) and the conditioned rewarding effects of morphine in male and female mice. More specifically, acute acetic acid-induced noxious state decreased the potency of morphine to function as a discriminative cue selectively in male but not in female mice. Further, these effects appeared to be dependent of the type of prescription opioid being examined as the discriminative stimulus effects of an alternative prescription opioid, oxycodone, did not vary as a function of pain in male and female mice. To the contrary, the conditioned rewarding effects of morphine were unaltered in the presence of acetic acid-induced noxious state in both male and female C57Bl/6 mice.

Morphine was effectively established as a discriminative cue with equivalent potency and efficacy in male and female C57Bl/6 mice using the two-choice operant drug discrimination procedure. Although this report is the first published morphine discrimination in an inbred mouse strain, these findings are consistent with previous studies in other species such as the rats, pigeons, and monkeys (237, 238, 240, 248). Sex differences in the discriminative stimulus effects of morphine have been studied to a lesser extent and only in rats. The findings from Craft et al. (241-243) are that morphine functions as a more potent discriminative cue in females *versus* males which is in contrast to the current study. In the series of studies by Craft et al. (241-243), the investigators

identified that the sex differences in morphine's discriminative effects were primarily attributed to differences in the reinforcement frequency (bias to saline lever in male *vs* female rats) rather than due to differential pharmacokinetics and sex-specific gonadal functions. Therefore, potential dissociable factors contributing to the differences between the current study and studies by Craft and colleagues include primary differences in species and the absence of reinforcement frequency bias in male *versus* female mice.

Oxycodone drug discrimination studies are poorly represented in the preclinical literature. In the current study, oxycodone dose-dependently and completely substituted for morphine in both sexes, but at slightly lower doses in males (about 2.5-fold more potent in males). Similar to the present results, oxycodone generalization to other mu-opioid receptor agonists, heroin and fentanyl have been previously demonstrated in male rats (203, 249), and to date, there are no previous studies that have characterized oxycodone's discriminative effects in female species. Similar to the observations in male mice in the present study, oxycodone was slightly more potent in its discriminative stimulus properties compared to morphine in male rats (249). Additionally, these preclinical findings are in parallel with comparable human laboratory studies that have characterized and demonstrated equivalent abuse liability and subjective measures for oxycodone and morphine in non-drug abusing and opioid-abusing populations (230, 232, 250).

A major finding in the present study is that acetic acid attenuated morphine-induced discriminative effects (decreased morphine's potency by 2.2-fold), particularly at lower morphine doses (0.1-3.2 mg/kg), without altering the efficacy of morphine's discriminative effects in male but not in female mice. Although some of the modulatory

effects of acetic acid on morphine's discriminative effects became apparent in the first discrete test trial session (15-20 min post morphine+acetic acid administration) (data not shown), several conflicting variables masked the interpretation of the selectivity of the modulating effects of acetic acid on morphine's discriminative cues during this time point. Specifically, during the first discrete test trial, morphine and acetic acid administration resulted in significant rate-decreasing effects in mice, perhaps because mice were also engaged in greater stretching behavior induced by acetic acid during this time frame. During the second discrete test trial session (35-40 min after morphine+acetic acid administration), there were no observed differences in the rates of responding compared to morphine alone (baseline) conditions. The number of acetic acid-induced stretches had significantly decreased, and morphine's discriminative cues still persisted without any significant changes from the observed effect during the first trial. Therefore, the data as represented from the second discrete test trial suggest that the modulatory effects of acetic acid are produced by a selective interaction between the acetic acid-induced noxious stimulus (nociceptive state) and morphine's discriminative cues, rather than from a general time-dependent decrease in morphine's cues or other non-selective behavioral consequences in male mice. It is well-established that morphine drug cues are mediated by central mu-opioid receptors (247, 251), further suggesting that the interaction between the drug cue and the acute noxious stimulus may also be occurring in the CNS.

In contrast to morphine, oxycodone's discriminative effects were unaltered in the presence of acetic acid-induced noxious state in both male and female mice. A possible interpretation of these data is that the opioid-type-selective effects of the acute noxious

stimulus on the discriminative cues of prescription opioids may be attributed (in part) to the differential intrinsic effectiveness of these opioids in reversing the acute noxious state in male and female mice (data shown in the previous chapter). That is, male mice revealed reduced sensitivity to morphine's antinociceptive effects in the acetic acid-induced stretching assay, particularly when morphine+acetic acid were co-administered at the lower doses (0.1-3.2 mg/kg) (procedure identical to the current drug discrimination study), relative to female mice (See figure 3, panel A, Chapter 2), while no potency differences were observed for oxycodone's antinociceptive effects between sexes (See figure 3, panel B, Chapter 2). The decreased sensitivity of male mice to morphine-induced antinociceptive effects at doses 0.1-3.2 mg/kg may have produced a more intense acetic acid-induced nociceptive state as compared to females, that may in part have contributed to the significant attenuation of the discriminative cues of morphine as observed at the identical dose conditions (0.1-3.2 mg/kg). As discussed in chapter 2, potential mechanistic differences underlie the differences in the antinociceptive effectiveness of morphine *versus* oxycodone in males and females. For example, sexual dimorphism exists in relation to the opioid receptor selectivity of these two agonists (morphine - predominantly mu-receptors *versus* oxycodone - putative kappa/mu activity) (155, 204); and/or evidence for sex-specific pharmacokinetic profiles and differential antinociceptive effectiveness of the metabolites of the two opioids in animals and humans (201, 211, 216, 252). These sex-specific factors that control the presence and/or the intensity of pain may consequently contribute to the differential modulatory influence of putative pain-states on the behavioral effects of opioids in males *versus* females, such as the discriminative stimulus effects noted in the present study.

The results of the present study are similar to the findings from previous human laboratory studies where the effects of experimentally induced pain on the subjective and abuse liability measures of prescription opioids such as morphine, oxycodone, and butorphanol were examined. More specifically, Conley et al. (110) demonstrated experimentally induced pain (cold pressor test) attenuated morphine-induced subjective effects (“high”, “coasting”), predominantly in male volunteers without any history of opiate dependence, while other studies in drug abusers, non-drug abusers, and recreational prescription opioid users showed that the subjective measures for oxycodone did not vary as a function of pain, although with comparable positive subjective ratings (“high”, “liking”, good effects) observed between the groups (73, 111).

Mechanisms underlying the sex-dependent modulatory effects of a putative pain-state on the discriminative stimulus effects of prescription opioids are largely unknown, however, one obvious possibility is for the modulatory effects to be mediated by a sexually dimorphic brain region that is known to regulate both nociception and the stimulus properties of opioids. For example, studies have drawn attention to a sexually dimorphic brain region, parabrachial nucleus (PBn), a visceral noxious stimuli relay station that is differentially engaged during visceral noxious stimulation and mu-opioid receptor mediated activation (via morphine) of this pathway in male *versus* female rodents (253, 254). The same brain region, PBn, in addition to the ventral tegmental area (VTA) and the periaqueductal gray (PAG) partially regulated the discriminative stimulus effects of morphine in rats (247, 251, 255, 256). Connecting these previous findings together with the behavioral data in the present study suggest that perhaps the sexually-dimorphic PBn region may regulate the modulation of morphine’s discriminative cues by

visceral nociceptive stimuli (such as the acetic acid-induced visceral nociception used in the current study) differentially in males *versus* females. Clearly, further studies are warranted to advance these findings and delineate the underlying mechanisms.

The present study findings expand the existing literature demonstrating morphine conditioned place preference in rodents (76, 235, 257). In naïve C57Bl/6 mice, females revealed a trend toward increased sensitivity to morphine-induced rewarding effects at the lower morphine doses (up to 1 mg/kg) compared to male mice; these results being similar to previous findings in rats (76, 235). However, at the higher doses (5 and 10 mg/kg), the magnitude of morphine-induced CPP was higher in male mice (although not statistically significant), relative to female mice and these results contrast with the findings in rats (235). The inconsistencies across studies could be attributed to species differences, routes of drug administration, and experimental conditions. The rewarding effects of morphine were fairly dose-independent and less consistent across the experiments in female mice in the present study. Previous studies have demonstrated that the rewarding and reinforcing effects of opioids vary as a function of estrogen status in female rodents (81, 258). For example, ovariectomized mice revealed decreased morphine CPP compared to gonadally intact female mice, and the effect was reversed by estradiol treatment in the ovariectomized mice (258). Therefore, as only gonadally intact mice were tested in the present study, it is possible that the increased variability observed in morphine CPP data in the present study in female mice may have been due to modulatory effects of the cycling hormones.

In the present study, acetic acid-induced noxious state failed to alter the conditioned rewarding effects of morphine in male and female mice. A small trend for an

increase in sensitivity to morphine's rewarding effects in the presence of the acute noxious state was observed in female mice. This apparent effect of pain-induced enhancement in morphine reward in female mice (observed as a significant CPP of the 2.5 and 10 mg/kg doses) was reflected by a relative change in the magnitude of CPP compared to the small place aversion to the drug-paired compartment observed in the vehicle conditioned group. However, there was no statistically significant effect of acetic acid treatment on morphine reward as confirmed using a two-way analysis of variance test. The results of the present study for female mice are in agreement with a previous study published by Bardin et al. (259) in male rats using the formalin-induced pain model. In rats, an increased morphine-induced reward was reported in the presence of pain and based on their experimental study design, the authors attributed the apparent increased morphine place-preference to the potential place-aversion to the alternative side of the CPP apparatus in the pain-group.

A possible explanation for the overall ineffectiveness of acetic acid on morphine reward is that the acetic acid-induced noxious stimulus fails to modify activation of the brain regions that contribute to reward by morphine, particularly the ventral tegmental area (VTA) of the midbrain which is well-established in mediating morphine-induced reward (113, 260). In support of this, a study by Bajic and Commons (261) revealed that morphine-mediated activation of the dopaminergic projection from the VTA were unaltered in the presence of acute chemical formalin-induced noxious stimulus. However, the applicability of this mechanism to acute acetic acid-induced noxious stimulus remains to be tested.

In summary, the present study established the discriminative stimulus effects of the prescription opioid morphine and substitution by oxycodone, another prescription opioid, in male and female C57Bl/6 mice. While morphine was equipotent in its discriminative properties in both sexes, oxycodone functioned as a more potent discriminative cue in male compared to female mice. Importantly, the present study revealed that an acetic acid-induced acute noxious state can preferentially attenuate the discriminative stimulus effects of morphine in male mice, but retain the conditioned rewarding effects of morphine in both sexes. The magnitude of modulation of the discriminative cues of opioids by acetic acid-induced noxious state were further found to be dependent (in part) on the relative intrinsic antinociceptive effectiveness of the two prescription opioids (inverse relationship) and on the sex-specific sensitivities of mice to opioid-induced antinociception. Further, the present study highlights the importance of assessing different aspects of the abuse-related measures of opioids (conditioned reward *versus* stimulus properties) as clearly distinct opioid-induced behavioral measures that are differentially modulated in the context of pain. It was surprising to note that a number of the present findings have not been documented in the preclinical literature, including establishment of the discriminative stimulus properties of opioids both in the presence and absence of pain in C57Bl/6 mice, and evaluation of the conditioned rewarding effects of morphine in the presence of acute pain dependent on sex. Future studies should be directed toward continued and systematic evaluation of the sex-dependent modulation of different opioid-induced behavioral effects in the presence of pain and subsequent identification of the mechanisms underlying these sexually-dimorphic effects.

CHAPTER 4
EFFECTS OF PACLITAXEL-INDUCED CHRONIC PERIPHERAL NEUROPATHY
ON THE DISCRIMINATIVE STIMULUS PROPERTIES, CONDITIONED
REWARDING, AND REINFORCING EFFECTS OF PRESCRIPTION OPIOIDS
IN MALE AND FEMALE MICE

Rationale

Chronic pain is a major public health problem that significantly diminishes the quality of life for patients (2, 3). From a clinical perspective, the use of prescription opioids for the management of chronic persistent pain is controversial due to the increased concern about tolerance, dependence, and addiction often associated with the long-term use of these drugs (47, 48, 99, 262-266). The prevalence of prescription opioid abuse among individuals who are prescribed opioids for the management of chronic pain has been estimated to be between 18– 45% (86, 99, 264, 267), although the true incidence is still largely unknown (94, 97, 268). Several factors have been identified as risk predictors for prescription opioid misuse or abuse among pain patients such as: personal and/or family history of substance use disorders, medication-related variables (exposure to prescription opioids and duration of therapy), psychological disorders and socio-economic, genetic, and environmental factors (269-271). Although the incidence of prescription opioid abuse and dependence are common among both men and women, gender-specific risk factors do exist (e.g. women more likely to use prescription opioids to cope with negative affect and psychiatric distress) (104-106). Despite this pervasive

problem, the modulatory role of chronic pain in the predisposition to prescription opioid abuse and the sexual-dimorphism in these behaviors are poorly understood.

Human laboratory studies suggest that in individuals who do not abuse drugs the reinforcing effects of prescription opioids are maintained in the presence of pain and that the subjective effects of some of these prescription opioids may be selectively attenuated in the context of experimentally induced pain (73, 109). These findings are consistent with the clinical studies reporting significant opioid self-administration in the presence of pain using patient-controlled analgesia procedures (112, 272-274) and suggest that in non-abusers opioids are not reinforcing as long as the pain is effectively alleviated. However, in contrast to the modulatory effects of pain on opioid self-administration in non-abusers, studies have demonstrated that opioid-abusers self-administer prescription opioids (e.g. oxycodone) regardless of the presence or absence of pain and the abuse liability of prescription opioids does not vary as a function of experimentally induced pain in this subject population (73, 111). While the findings in non-abusers do not support the human clinical reports (increased prevalence of opioid misuse in chronic pain patients), as discussed above, findings in opioid-abusers suggest that perhaps the history of opioid use/drug-use status of an individual is an important variable in predicting predisposition to prescription opioid abuse among chronic pain patients.

Preclinical studies have advanced the understanding of neuroanatomical and molecular changes that may be associated with the development of persistent chronic pain in animals. For example, supraspinal molecular alterations underlying chronic pain have been demonstrated to occur both in the pain-related and reward-related brain regions (275) including the ventral tegmental area (VTA) (113, 276-278), nucleus accumbens (114,

279), and amygdala (115, 280, 281). For these reasons, several studies have examined the modulatory effects of chronic pain on the behavioral effects of opioids using different animal models of addiction, the findings being mixed and demonstrating either an increase (116-118, 282-284) or a decrease in the rewarding and reinforcing effects of opioids in the presence of a chronic pain-like state in rodents (113, 117, 119, 285-288). For example, arthritic rats self-administered greater amounts of fentanyl compared to pain-free animals (118), surgery-induced neuropathic pain in rats maintained self-administration of opioids at doses that effectively reversed mechanical hypersensitivity (117), and opioid-induced place preferences were enhanced in the presence of persistent inflammatory and chronic neuropathic pain in rats (116, 282, 284). These data suggest that the primary motivation for opioid-seeking is directly related to pain relief and largely support the clinical findings in humans (73, 109, 272, 273). However, other studies reported that the arthritic rats self-administered fewer infusions of morphine compared to normal rats (119), the presence of neuropathic pain and persistent inflammatory pain decreased the conditioned rewarding effects of morphine (113, 285), and the ability of prescription opioids to facilitate the stimulation of the VTA are attenuated following peripheral nerve injury in rats (as determined using the intracranial self-stimulation procedures) (287, 289). Therefore, a number of factors may influence the manner in which chronic pain-states could alter the reward-related functions of opioids, including the model of chronic pain, type of opioid studied, dose used, route of administration, type of animal model of addiction used, and/or a combination of these factors. While the majority of these studies have been reported in rats, only in males, and limited to a few behavioral assessments in any given chronic pain model, a number of other factors such

as the role of sex, and other measures of opioid abuse liability (e.g. discriminative stimulus properties) in the context of paclitaxel-induced chronic pain (which is relatively less studied) have not been investigated.

It is well-established that the administration of taxane-family chemotherapeutics such as paclitaxel in rodents damages peripheral sensory neurons, including neuronal mitochondrial and axonal transport dysfunction, altered signal conduction, and distal axonal degeneration (145, 188-190). The persistent behavioral manifestations of these peripheral sensory alterations include reduced threshold and increased responsiveness to non-noxious stimuli (allodynia) as demonstrated previously by our laboratory in C57Bl/6 mice (147, 148) (discussed in chapter 2). The current study involved a comprehensive side-by-side assessment of the effects of chronic paclitaxel-induced peripheral neuropathy on three aspects of prescription opioid-reward in male and female C57Bl/6 mice namely, discriminative stimulus properties (using a drug discrimination assay), conditioned rewarding effects (using a CPP procedure), and the reinforcing effects (using a progressive ratio schedule in the intravenous self-administration assay). A range of low to high doses of the two prescription opioids, morphine and oxycodone were tested in the drug discrimination and conditioned place preference studies in order to determine the sex-specific, dose-effect relationship on the modulatory effects of chronic pain on opioid reward. The results from the conditioned place preference studies guided the testing of morphine in the self-administration assay in an effort to elucidate potential sex differences in reinforcing efficacy and opioid-seeking behaviors measured in terms of a number of variables that are representative of motivation (breakpoints- defined in

methods), drug consumption (morphine intake), and drug taking patterns in the presence of a developing chronic pain state in mice.

Methods

Subjects

Male C57Bl/6 mice weighing 20-25 g, and female mice weighing 15-20 g, and all mice 5-6 weeks of age, were purchased from SAGE Labs (Boyertown, PA, USA) for the drug discrimination (N=11, males; N=11, females) and self-administration studies (N=20, males; N=20, females), and from Taconic, Farms, Inc. (Cranbury, NJ, USA) for the conditioned place preference experiments (N=109, males; N=107, females). Mice were group housed in plastic cages and allowed to acclimate to the temperature- and humidity-controlled animal facility for 3 to 7 days before the experiments began. Mice were housed under a 12 h light/dark cycle with lights off at 10.00 h, so that all experiments occurred during the dark phase of the diurnal cycle. For the conditioned place preference studies, mice were housed in groups of four and food and water were available *ad libitum*. For the drug discrimination studies, mice were individually housed a day prior to commencement of the experiment and maintained on 90% of their free feeding body weights (approximately 2.75 g pellet daily of Purina Rodent Chow Diet 5001 (Ralston-Purina, St. Louis, MO, USA)) for the remainder of the study. For the self-administration studies, mice were individually housed a day prior to commencement of the experiment and maintained at 90% of their free-feeding body weights (approximately 2.75 g pellet daily of Purina Rodent Chow Diet 5001 (Ralston-Purina, St. Louis, MO, USA)) during the initial food pre-training phase, and subsequently received food and water *ad libitum*

for the remaining operant experiments. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University and the Guide for the Care and Use of Laboratory Animals (Institution of Laboratory Animal Research, National Academy Press; Eighth edition, revised 2011).

Drugs

Morphine sulfate and oxycodone hydrochloride were generously donated by the National Institute on Drug Abuse (NIDA) drug supply program (Bethesda, MD, USA). Paclitaxel (dissolved in a 1:1 mixture of alcohol and Cremophor) was obtained as a 6 mg/mL concentration stock solution (Hospira, Inc., Lake Forest, IL, USA). Morphine, oxycodone, and paclitaxel were dissolved in 0.9% saline. All injections were administered intraperitoneally (IP) in a volume of 0.01 ml/g of body weight.

Experiment 5: Opioid Drug Discrimination

Apparatus

Experiments were conducted in twelve chambers (21.6 cm x 17.8 cm x 12.7 cm, Model ENV-307W, Med Associates, Georgia, Vermont, USA) located within ventilated sound attenuating enclosures. One wall of the chamber contained two nose poke holes (left and right: 1.2 cm diameter) with internal amber stimulus lights (ENV-313W) and two amber stimulus lights mounted on the wall directly above the two nose-poke holes. A food receptacle (center dipper hole) was present between the two nose pokes that opened to a motor-driven dipper (ENV-302W) for liquid food presentation. The opposite wall of the chamber contained a house light (ENV-315M), tone generator (ENV-

323AW), and ventilator fan that generated white noise. The receptacle for dipper access contained an amber stimulus light located above it (ENV-221M). All experimental contingencies were arranged, and data were recorded by a computer-driven interface (Model SG-503, MED Associated, St. Albans, VT, USA).

Procedure

Mice were trained to discriminate saline or morphine (3.2 mg/kg) using a two-choice two-trial operant procedure adapted and modified from Walker and Young (237). Description of the procedure for training, establishment of stimulus cues for the training drug morphine, and generation of dose-response curves for the two prescription opioids morphine and oxycodone, are detailed in the procedure section of chapter 3.

Effect of chronic paclitaxel-induced peripheral neuropathy on opioid discrimination. After the initial dose-response curves for morphine and oxycodone were generated in different groups of male and female C57Bl/6 mice, mice were randomly assigned to saline or paclitaxel treatment groups. Mice received saline or paclitaxel (8 mg/kg) injections on a standard dosing regimen on days 1, 3, 5, and 7 (147). Repeated and alternative training on saline and drug cues were suspended in all groups of mice during the one week dosing of saline or paclitaxel (X 4 injections, every other day) primarily to minimize confounds that may arise from the interaction between the training, reinforcing changes in the discriminative cues produced by paclitaxel, and the developing chronic pain state. Following treatment, the dose-response curves for morphine and oxycodone were re-established during the period of peak allodynia (period of increased

sensitivity to non-noxious mechanical stimulus, between days 11 to 19 post 1 injection of paclitaxel) (147). The potency of morphine and oxycodone's discriminative effects were determined and compared between the saline and paclitaxel-treated groups to assess the discriminative effects of the two prescription opioids in the presence of paclitaxel-induced chronic peripheral neuropathic pain state.

Data and Statistical Analysis

ED₅₀ values were calculated for every mouse in the two groups of male and female mice using linear regression analysis (Graphpad Prism 5.0 software) and averaged into group means and compared using unpaired Student's t-test prior to starting treatment with saline or paclitaxel. Subsequently, to determine the effects of paclitaxel-induced chronic neuropathy on the discriminative stimulus effects of opioids in mice, individual ED₅₀ values were calculated for every mouse from the linear portion of dose-response effect curve for the two prescription opioids post-treatment using linear regression analysis (Graphpad Prism 5.0 software). The individual ED₅₀ values were averaged into group means and compared between the saline- and paclitaxel-treated groups using unpaired Student's t-test within each sex. Statistical significance was set at P<0.05 for all analyses.

Experiment 6: Opioid Conditioned Place Preference (CPP)

Apparatus

The CPP apparatus consisted of 8 identical experimental chambers (Model ENV-3013, MED Associates, St. Albans, VT, USA), each with white (white walls and mesh

flooring), middle grey, and black (black walls and bar flooring) compartments containing distinct cues and light settings. Each chamber was located within a sound-attenuating enclosure and connected to a computer-driven interface (Model SG-6080/D, MED Associates, St. Albans, VT, USA) that controlled the data collection and experimental conditions.

Procedure

A pseudo-biased place-conditioning procedure as defined and described in chapter 3 was incorporated in the following studies. For a detailed description of the paradigm, refer to the procedure section of chapter 3.

Effect of chronic paclitaxel-induced peripheral neuropathy on opioid-induced conditioned reward. Male (n = 109) and female (n = 107) C57Bl/6 mice were divided into control and treatment groups. The control group of mice received saline injections and the treatment group received paclitaxel (8 mg/kg) injections on a standard dosing regimen on days 1, 3, 5, and 7 (147). Starting on Day 11, after the first paclitaxel drug administration, during the period of peak allodynia (increased sensitivity to a non-noxious mechanical stimulus), mice were conditioned by alternating between saline (vehicle) and one of the drug doses (morphine: 0.3, 2.5, or 10 mg/kg; oxycodone: 0.3 or 10 mg/kg) for three pairings and tested for their preference for the previous drug-paired environment in a drug-free state post-conditioning sessions (day 7). Additionally, separate groups of saline- and paclitaxel-treated mice were also conditioned by alternating with vehicle alone (saline) on both sides of the CPP compartments. The effect

of paclitaxel treatment on opioid-reward was determined by comparing the preference for the drug-paired compartments between the control saline-treated and paclitaxel-treated groups of mice within each sex. The morphine doses were identical to the doses of morphine tested in the acute pain studies (as described in chapter 3) to directly compare the effects of chronic paclitaxel-induced neuropathy to acute acetic acid-induced noxious state on morphine reward in mice. Further, the present study was extended to assess the effects of chronic paclitaxel-induced neuropathy on oxycodone reward (two doses tested: a non-rewarding dose (0.3 mg/kg) and a rewarding dose (10 mg/kg)) to compare the two prescription opioids.

Data and Statistical Analysis

Time spent in each compartment in a drug-free state was recorded on the test day and used to calculate the preference score for each mouse using the following equation:

$$\textit{Preference score} = [\textit{Time spent in the morphine-paired compartment}] - [\textit{Time spent in the saline-paired compartment}]$$

Separate one-way ANOVAs (GraphPad Prism 5.0 Software, Inc, La Jolla, CA) with Tukey's multiple comparison posthoc tests were used to determine the sensitivities to morphine-induced conditioned rewarding effects within control and paclitaxel-treated groups of male and female mice. To determine the interactions between and the factors Treatment and Dose within each sex, two-way ANOVAs were used with Bonferroni posthoc tests. Statistical significance was set at $p < 0.05$ for all analyses.

Experiment 7: Morphine Self-Administration

The reinforcing effects and the motivational property of the prescription opioid, morphine and opioid seeking behaviors were assessed in the presence of paclitaxel-induced chronic neuropathy in male and female mice using an intravenous morphine self-administration assay. Male and female C57Bl/6 mice were trained to respond for food in the operant chambers. Next, morphine intravenous self-administration was established initially under a fixed ratio (FR1) schedule of reinforcement that was followed by morphine self-administration under a progressive ratio (PR) schedule of reinforcement using a progressive ratio series adapted from Richardson and Roberts (130). Details of the food pre-training, acquisition of morphine self-administration (FR1 and PR), and test phases of the assay are outlined below:

Apparatus

Food pre-training and morphine self-administration experiments were conducted in standard mouse operant conditioning chambers (21.6 cm x 17.8 cm x 12.7 cm, Model ENV-307W, Med Associates, Georgia, Vermont, USA). The operant experimental chambers were located within ventilated sound attenuating enclosures and each chamber was equipped with the following: two nose-poke holes- left and right (1.2 cm diameter) with internal amber stimulus lights (ENV-313W), center dipper hole between the two nose-pokes that opened to a motor-driven dipper (ENV-302W) for liquid food presentation, a house light (ENV-315M), tone generator (ENV-323AW), and ventilator fan. The receptacle for dipper access contained an amber stimulus light located above it (ENV-221M). In addition to the features explained above, morphine self-administration chambers contained an electronic circuit that operated a computer-controlled syringe

pump designed for intravenous drug-delivery. The syringe was connected to a single-channel fluid swivel mounted on a counter-balanced arm above the operant chamber (MED-307A-CT-B2).

Procedure

Food pre-training. A day prior to commencement of the experiment, mice were singly housed and their free-feeding body weights were recorded in order to maintain the mice at 90% of their free-feeding body weights during the remainder of the experimental period. This corresponded to approximately 2.5 g pellet feed daily of Purina Rodent Chow Diet 5001 (Ralston-Purina, St. Louis, MO, USA). Starting from Day 1, separate groups of food-restricted male and female C57Bl/6 mice were trained to respond on the right nose-poke for a 50% Ensure solution under a Fixed Ratio (FR1) schedule of reinforcement during daily 1 h food pre-training sessions in the operant experimental chambers. During each session, every response in the right illuminated nose-poke hole resulted in illumination of the adjacent light and delivery of liquid food through the center dipper receptacle for 20 s. In addition to the number of reinforcers earned during each session, the number of inactive nose-poke responses and the total number of head entries into the dipper receptacle were also recorded, although these responses had no scheduled consequences during the experimental sessions. The criterion for acquisition of liquid food self-administration was defined as three consecutive days of stable FR1 responding (<10% changes from the mean number of reinforcers earned for the three days) and with at least 75% of the total responses corresponding to the active right nose-pokes. Following food acquisition (approximately 1 week), mice were given free access to food and water as they proceeded through the remaining operant studies.

Acquisition of morphine self-administration: dose-response effect (FR1).

Individual mice that met criteria for food self-administration acquisition were surgically implanted with a chronic indwelling jugular cannula using a procedure described by Caine et al. (290). Following surgery and 2 days of recovery, all mice were trained to self-administer 0.1 mg/kg/infusion morphine under an FR1 schedule of reinforcement during daily 2 h sessions. This initial training dose (0.1 mg/kg/inf) for morphine was chosen based on a study reported in the literature showing that in the C57Bl/6 mouse strain, morphine maintained maximal response rates at this dose under a fixed ratio schedule of drug reinforcement (291). During the session, every response on the active right illuminated nose-poke resulted in a single infusion of morphine paired with illumination of the light above the food receptacle and a tone delivery for 1 s. After every morphine infusion, the houselights went off for 60 s in the chambers and the mouse's response had no programmed consequence during this period. After stable responding under an FR1 schedule defined by the same criteria outline above in the food pre-training section, a dose-response curve was generated in male and female C57Bl/6 for a range of morphine doses (0.01-0.3 mg/kg/inf).

Morphine self-administration: maintenance and progressive ratio responding.

Based on the results of the dose-response effects observed for morphine self-administration under an FR1 schedule of responding in male and female mice, separate training doses were chosen for the subsequent experiments for male (0.1 mg/kg/inf) and female (0.03 mg/kg/inf) mice. These doses represented the preferred doses of morphine for males and females, respectively, at which morphine self-administration occurred in

about 7 d. After stable responding under the FR1 schedule of responding at their respective training doses, male and female mice were given access to morphine (at the same corresponding training doses) under a PR schedule of reinforcement. In this schedule, the response requirements to earn a single reinforcer were increased exponentially in the following progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc. as described in Richardson and Roberts (130). The dependent variable measured was the 'breakpoint', defined as the number of infusions earned by the end of the 4 h session. Total cumulative responses made by the mouse on the correct active-hole (right) nose-poke during each 4 h session were also recorded. The criterion for stable responding under the PR schedule was defined as three consecutive days of stable breakpoints (<20% change from the mean number of reinforcers earned for the three days). Mice that did not acquire PR responding or produced very low breakpoints (lower than 4 infusions) [this corresponded to 1 female and 2 male mice] and those mice whose catheters blocked after starting saline or paclitaxel treatment [this corresponded to 1 female and 3 male mice], were not included in the study.

Effect of chronic paclitaxel-induced peripheral neuropathy on morphine self-administration maintained on a progressive ratio schedule of responding. Following stable responding on the PR schedule (~ 1 week), mice were randomly assigned to receive saline (control) or 8 mg/kg paclitaxel (treatment) injections on a standard dosing regimen on days 1, 3, 5 and 7 (147) while continuing to respond under a PR schedule of responding during daily self-administration sessions. Mice received injections every other day after completion of the 4 h PR session. PR responding for morphine, at their

respective training doses (males – 0.1 mg/kg/inf; females – 0.03 mg/kg/inf), were recorded during the development of the allodynia period (between days 0 and 11) and compared between the saline and paclitaxel treatment groups within each sex. The experiment continued up to day 35 after the first paclitaxel injection (~ 5 weeks). Between days 11 and 20 after the first paclitaxel drug administration (corresponding to the period of peak allodynia), mice were tested for their PR responding for morphine at three doses (0.01 to 0.1 mg/kg/inf). On days 11, 12, and 13, mice were tested with their training doses (males - 0.1 mg/kg/inf; females – 0.03 mg/kg/inf). The subsequent two test conditions for the remaining two doses of morphine were counter-balanced within each sex and the saline/paclitaxel treatment groups. The breakpoints measured on the three test days for each of the three doses of morphine were averaged for each mouse and then averaged into group means and compared between saline- and paclitaxel-treated groups. Following the behavioral testing, mice (still retaining catheter patency) were given access to saline under the PR schedule for 10 d and their responding under extinction conditions was assessed.

Data and Statistical Analysis

The dependent variable for morphine self-administration under an FR1 schedule was the response rate or the number of infusions earned in 2h sessions. Separate one-way ANOVAs (GraphPad Prism 5.0 Software, Inc, La Jolla, CA) with Tukey's multiple comparison posthoc tests were used to analyze the dose-response curves for morphine under the FR1 schedule in male and female mice. Two-way ANOVAs were used to determine the effects of the factors and the interactions between factors for the following

data-sets: sex and dose for the FR1 morphine self-administration data; treatment and day for the PR data for morphine during the allodynia development period within each sex and during extinction with saline within each sex; treatment and dose effects for the PR dose-response analyses within each sex. Percentage of mice showing an increase in breakpoints for morphine from baseline measures were calculated within saline and paclitaxel-treated male (at 0.1 mg/kg/inf) and female (at 0.03 mg/kg/inf) groups and compared. The response patterns maintained by morphine under the progressive ratio schedule representing the time of morphine infusions and inter-infusion intervals during daily 4 h sessions were defined by the event records captured using the SoftCR Pro software (SOF-722 SOFTCR PRO, MED Associates Inc, St Albans, VT). The event records were compared between saline and paclitaxel-treated mice within each sex in order to compare the response/drug taking pattern for morphine in the presence and absence of chronic pain. Conditions under which significant differences were noted in the pattern of responding for morphine or the total and time-dependent intake of drug were determined and compared between and within treatment groups using unpaired and paired Student's t-test. Statistical significance was set at $p < 0.05$ for all analyses.

Results

Experiment 5: Opioid Drug Discrimination in the Context of Chronic Pain

In the present study, two groups of male and two groups of female C57Bl/6 mice were trained to discriminate morphine from saline. Morphine was effectively established as a discriminative cue in both groups of male (Figure 17, panel A) and female C57Bl/6 mice (Figure 18, panel A) as the training dose (3.2 mg/kg) and higher doses produced

near- 100% morphine-appropriate responding in the two-choice operant drug discrimination procedure. Oxycodone fully substituted for morphine by producing greater than 80% morphine-appropriate responding at the highest dose tested (3.2 mg/kg) in both groups of male (Figure 17, panel A) and female mice (Figure 18, panel A).

Table 4. Summary of statistical analyses for the effects of paclitaxel-induced chronic neuropathy on the discriminative stimulus effects of morphine and oxycodone in male mice.

Groups	Baseline		Post-treatment	
	Morphine ED ₅₀ values ±SEM mg/kg (n = 5-6)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 5-6)	Morphine ED ₅₀ values ±SEM mg/kg (n = 5-6)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 2-5)
Group 1 (Sal)	1.5 ± 0.26	0.75 ± 0.27	2.8 ± 1.4	2.0 ± 0.05
Group 2 (Paclitaxel)	1.5 ± 0.23	0.80 ± 0.21	4.4 ± 1.2	1.5 ± 0.24

At baseline, the stimulus generalization patterns for morphine and oxycodone were identical across the trained groups of male (see ED₅₀ values for morphine and oxycodone for the two groups under baseline conditions in Table 4) and female C57Bl/6 mice (see ED₅₀ values for morphine and oxycodone for the two groups under baseline conditions in Tables 5).

Males

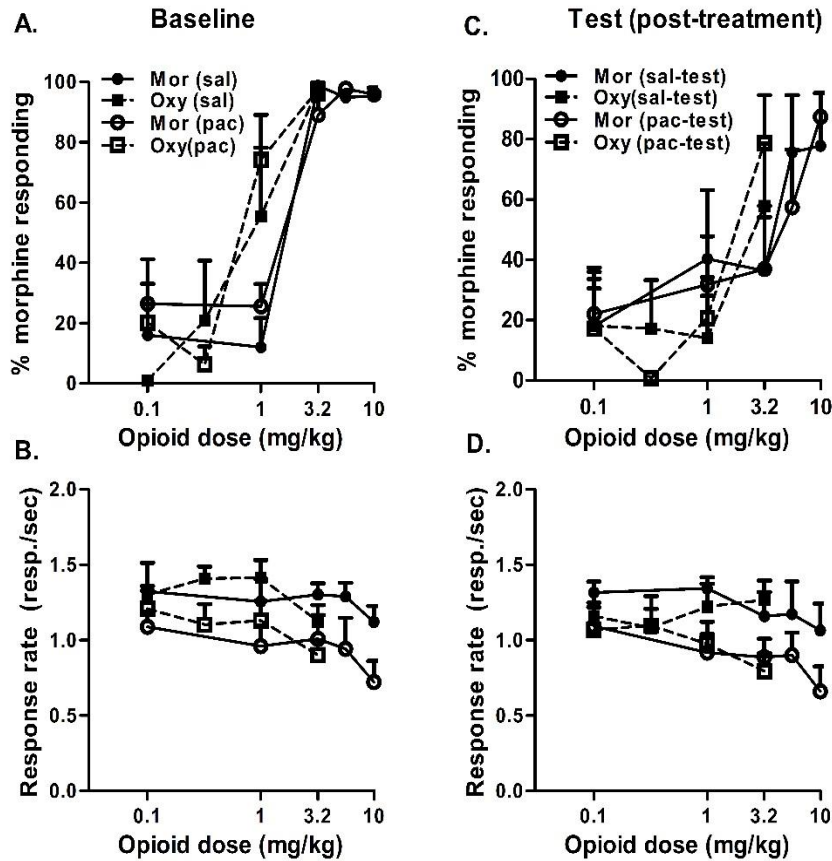


Figure 17. Discriminative stimulus and rate-decreasing effects of morphine in two groups: sal- mice assigned to saline treatment (filled circles and solid line); pac- mice assigned to paclitaxel treatment (open circle and solid line) and oxycodone in the same two groups (sal- filled squares and dotted line; pac- open squares and dotted line) at baseline (prior to treatment) - panels A and B and test (post-treatment) - panels C and D in $n = 5-6$ mice/group of C57Bl/6 male mice. Abscissa: Doses of the opioid expressed as mg/kg. Ordinate: % of total responses directed to the training drug (morphine)-appropriate nose poke (top panels A and C); Responses per unit time (resp./sec) (bottom panels B and D). All points represent the mean % of morphine-appropriate responding (panels A and C) or mean response rates (panels B and D) in the respective groups + SEM.

Table 5. Summary of statistical analyses for the effects of paclitaxel-induced chronic neuropathy on the discriminative stimulus effects of morphine and oxycodone in female mice.

Groups	Baseline		Post-treatment	
	Morphine ED ₅₀ values ±SEM mg/kg (n = 5)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 5-6)	Morphine ED _{50±} values SEM mg/kg (n = 5)	Oxycodone ED ₅₀ values ±SEM mg/kg (n = 3-6)
Group 1 (Sal)	1.4 ± 0.33	1.0 ± 0.35	4.0 ± 0.60	0.42 ± 0.12
Group 2 (Paclitaxel)	1.4 ± 0.34	0.83 ± 0.24	4.0 ± 1.1	0.88 ± 0.23

Paclitaxel-induced, chronic peripheral neuropathy failed to alter the discriminative stimulus effects of the two prescription opioids, morphine and oxycodone in either male (see ED₅₀ values for the two groups under post-treatment in Table 4) (Figures 17, panel C) or female mice (see ED₅₀ values for the two groups under post-treatment in Table 5) (Figures 18, panel C). Unpaired t-test analyses of the ED₅₀ values for the discriminative stimulus effects of morphine and oxycodone between the two treatment groups (saline vs paclitaxel), revealed no statistically significant differences ($P > 0.05$). Further, as seen from the tables (4 and 5), the potencies of morphine across both treatment groups of male and female mice, and oxycodone in male mice were decreased from baseline to post-test conditions (by about 2 to 3-fold), as represented by the rightward shifts in the dose-response curves for the two opioids (Figures 17 and 18, panel C). No differences were seen across groups post-treatment in the rate-decreasing effects of the two opioids (Figures 17 and 18, panel D).

Females

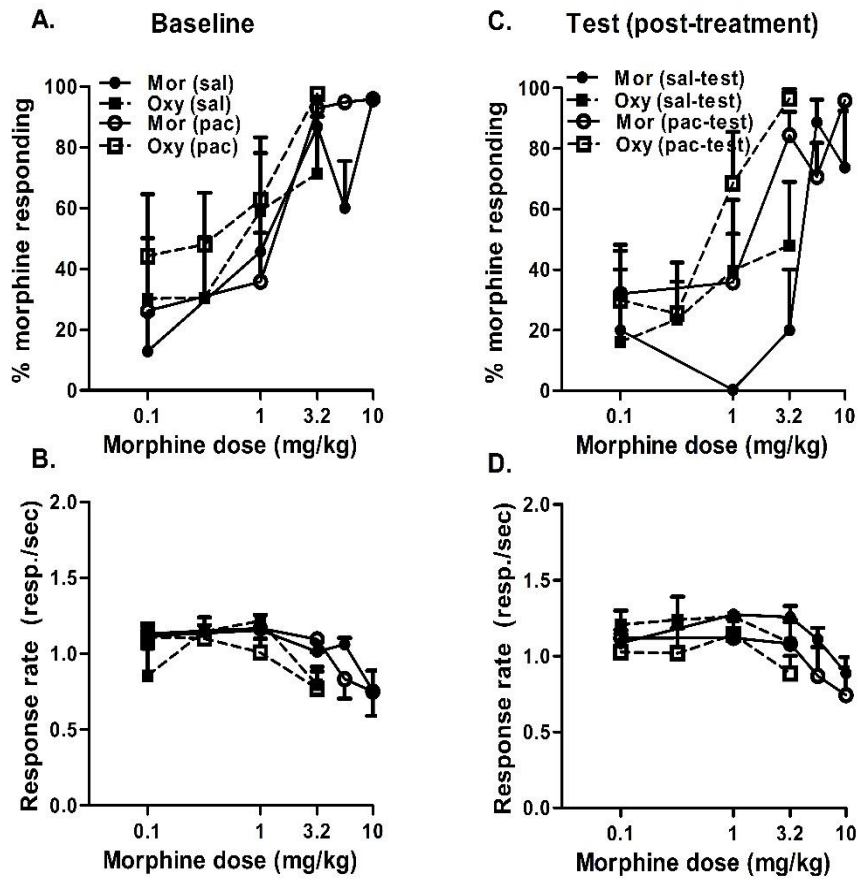


Figure 18. Discriminative stimulus and rate-decreasing effects of morphine in two groups: sal- mice assigned to saline treatment (filled circles and solid line); pac- mice assigned to paclitaxel treatment (open circle and solid line) and oxycodone in same two groups (sal- filled squares and dotted line; pac- open squares and dotted line) at baseline (prior to treatment) - panels A and B and test (post-treatment) - panels C and D in $n = 5-6$ mice/group of C57Bl/6 female mice. Abscissa: Doses of the opioid expressed as mg/kg. Ordinate: % of total responses directed to the training drug (morphine)-appropriate nose poke (top panels A and C); Responses per unit time (resp./sec) (bottom panels B and D). All points represent the mean % of morphine-appropriate responding (panels A and C) or mean response rates (panels B and D) in the respective groups + SEM.

However, the experiment was set-up and controlled for analyses only comparing between treatment groups at baseline and post-test conditions, but not within groups. Hence, analyses within groups were not performed but have been discussed (see discussion).

Experiment 6: Opioid Conditioned Place Preference in the Context of Chronic Pain

The conditioned rewarding effects of the prescription opioids, morphine and oxycodone were assessed in the presence of paclitaxel-induced chronic peripheral neuropathy using the conditioned place preference procedure in male and female mice. In the control (saline-treated) group, morphine produced significant dose-dependent rewarding effects in male and female mice ($p < 0.0001$) (Figure 19, panels A and B). Posthoc-tests revealed significant differences between the lower and higher doses of morphine in both sexes. Specifically, morphine-induced CPP was significantly different between the rewarding doses (2.5 and 10 mg/kg) and the sub-threshold non-reward dose (0.3 mg/kg) ($p < 0.05$) in the control male mice, while in the control female mice a significant difference was observed between 0.3 and 10 mg/kg doses of morphine ($p < 0.05$).

Paclitaxel treatment significantly enhanced the sensitivities of male and female mice to the conditioned rewarding effects of morphine (Figure 19, panels A and B). A one way ANOVA within the paclitaxel-treated male and female groups revealed a significant effect of dose ($p < 0.0001$). Tukey's multiple comparison posthoc tests revealed that all three doses of morphine tested (0.3-10 mg/kg) produced significant CPP compared to vehicle-conditioned (saline) mice within the paclitaxel groups in each sex. While the dose-dependency was no longer observed in the paclitaxel-treated male mice

(since morphine CPP at 0.3 mg/kg had increased in magnitude and was comparable to the higher doses), dose-dependency was maintained in the paclitaxel-female group with the 2.5 ($p < 0.05$) and 10 mg/kg ($p < 0.01$) morphine doses producing significantly greater CPP relative to the 0.3 mg/kg dose.

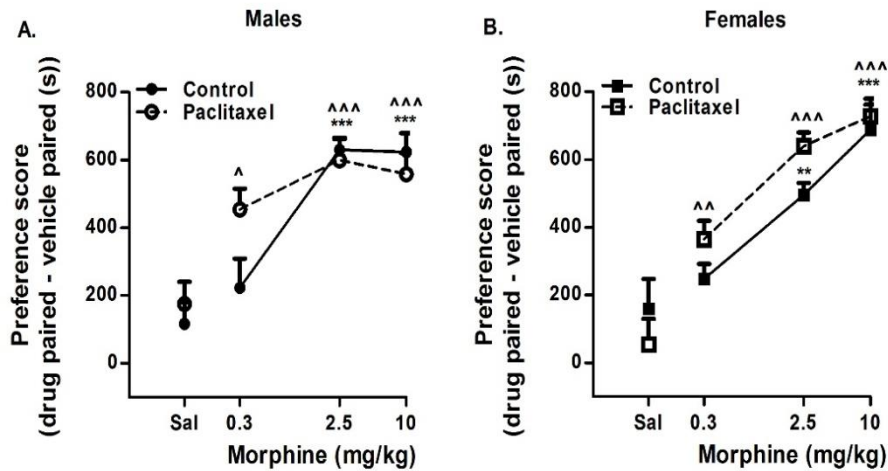


Figure 19. Effects of paclitaxel-induced chronic neuropathy on the conditioned rewarding effects of morphine in male (panel A) and female (panel B) mice. Abscissa: Control (Sal: saline), or doses of morphine expressed as mg/kg. Ordinate: Preference score (s): time spent in the drug - vehicle paired sides on the test day. Each point represents the mean preference score + SEM ($n = 8-16/$ group). **, $p < 0.001$, *** $p < 0.0001$: compared to saline (Sal) in the control group within each sex. [^], $p < 0.05$; ^{^^}, $p < 0.01$, ^{^^^}, $p < 0.0001$ compared to saline (Sal) in the paclitaxel group within each sex as determined by a one-way ANOVA.

In male mice, a two-way ANOVA revealed a significant main effect of dose [$F_{(2, 42)} = 11.96$, $p < 0.0001$], no main effect of treatment [$F < 1.0$], but a significant interaction [$F_{(2, 42)} = 3.35$, $p = 0.0448$] between dose and treatment effects (Figure 19, panel A). Further, Bonferroni posthoc tests revealed significant differences in morphine CPP between the saline and paclitaxel groups of male mice at the sub-threshold low dose of 0.3 mg/kg. A

two-way ANOVA revealed an overall significant main effect of dose [$F_{(2, 45)} = 30$, $p < 0.0001$], significant main effect of treatment [$F_{(1, 45)} = 5.39$, $p = 0.0248$], but no significant interaction [$F < 1.0$] in female mice (leftward and upward shift in the dose-response effect of morphine CPP, figure 19, panel B). However, the Bonferroni posthoc tests did not reveal any significant differences between the saline and paclitaxel-treated female groups at any dose of morphine tested.

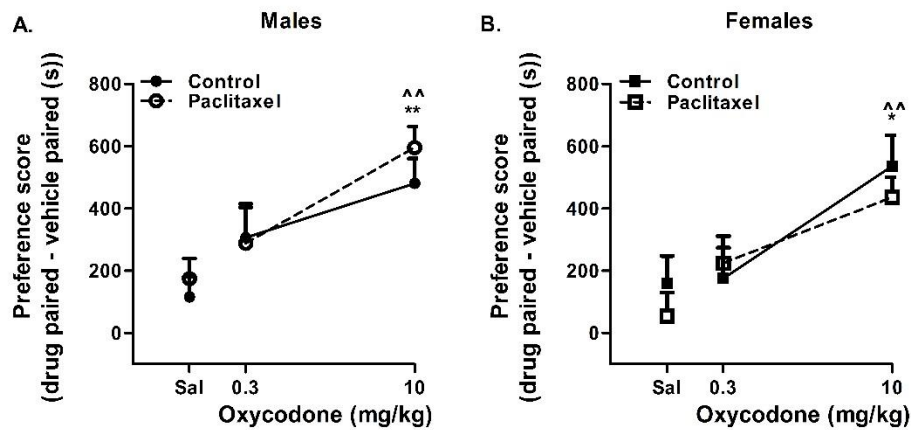


Figure 20. Effects of paclitaxel-induced chronic neuropathy on the conditioned rewarding effects of oxycodone in male (panel A) and female (panel B) mice. Abscissa: Control (Sal: saline), or doses of oxycodone expressed as mg/kg. Ordinate: Preference score (s): time spent in the drug - vehicle paired sides on the test day. Each point represents the mean preference score + SEM ($n = 7-16$ / group). *, $p < 0.05$: compared to saline (Sal) in the control group within each sex. ^^, $p < 0.01$, ^^ compared to saline (Sal) in the paclitaxel group within each sex as determined by a one-way ANOVA.

In contrast, paclitaxel-induced chronic neuropathy did not alter the sensitivities of male and female mice to the conditioned rewarding effects of an alternative prescription opioid, oxycodone. As determined by a one-way ANOVA within the control (saline-treated) and paclitaxel-treated male and female groups, oxycodone produced significant

rewarding effects only at the 10 mg/kg dose (male (control): $p = 0.014$; male (paclitaxel): $p = 0.0048$; female (control): $p = 0.0191$; female (paclitaxel): $p = 0.0084$) (Figure 20, panels A and B). A two-way ANOVA revealed a significant main effect of dose in male [$F_{(1, 26)} = 6.05$, $p = 0.0209$] and female [$F_{(1, 27)} = 10.04$, $p = 0.0038$] mice, but no significant main effect of the treatment or an interaction [$F < 1.0$] in either sex.

Experiment 7: Intravenous Morphine Self-Administration

The data presented in figure 21 illustrates an inverted-U shaped dose-response effect for morphine under an FR1 schedule of reinforcement in male and female C57Bl/6 mice. Morphine produced reinforcing effects in male and female C57Bl/6 mice. A one-way ANOVA revealed morphine at the 0.1 mg/kg/inf dose to be self-administered significantly more than saline in male mice ($p = 0.0237$). However, Tukey's multiple comparisons posthoc tests revealed no significant differences between the doses tested in males. In female mice, a one-way ANOVA revealed a significant main effect of dose ($p = 0.0003$) with the response rates at 0.03 and 0.3 mg/kg/inf morphine being significantly different from responding for saline. Further, significant differences were observed between the 0.03 and 0.1 mg/kg/inf doses of morphine in female mice as demonstrated by Tukey's posthoc tests. The peak effect observed for morphine between sexes was half a log unit apart (females: 0.03 mg/kg/inf and males: 0.1 mg/kg/inf). However, a two-way ANOVA revealed no significant effect of sex, dose [$F < 1.0$] or an interaction between the factors [$F_{(3, 61)} = 1.97$, NS].

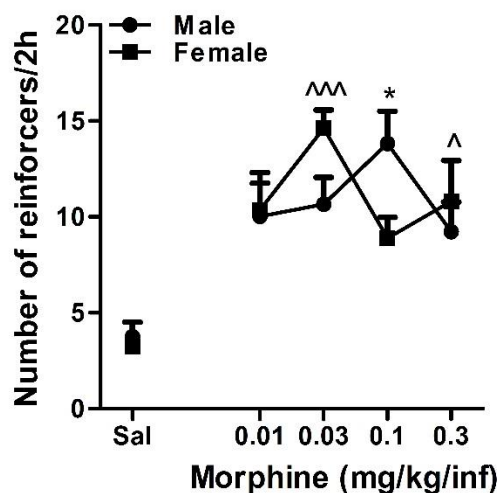


Figure 21. Responding for morphine under an FR1 schedule of reinforcement in male (filled circles) and female (filled squares) C57Bl/6 mice. Abscissa: Control (Sal: saline), or dose of morphine expressed as mg/kg/infusion. Ordinate: Response rate. Each data point represents the mean number of responses earned within each 2 h session + SEM (n = 5-18/group). *, p < 0.05: significantly higher response rates than sal-male group; ^, p < 0.05; ^^^, p < 0.01 compared to the sal-female group as determined by a one-way ANOVA.

Morphine supported an average breakpoint of 6.4 (\pm 0.32) across male mice at their respective training dose (0.1 mg/kg/inf) and a higher average breakpoint of 7.2 (\pm 0.27) across female mice at their training dose of 0.03 mg/kg/inf. Paclitaxel administration produced a significant change in responding for morphine under the progressive ratio schedule in male C57Bl/6 mice (Figure 22, panel A). Specifically, in male mice, the motivation to respond for morphine (0.1 mg/kg/inf) significantly increased over time in the paclitaxel-treated group compared to the saline-treated control group. This increase appeared to correlate with the development of allodynia on days post-paclitaxel administration (days 1, 3, 5, and 7), reaching higher breakpoints around the typical period of peak allodynia (days 11 to 20 post first injection of paclitaxel) (see

Table 6). However, a two-way ANOVA only revealed a significant main effect of treatment [$F_{(1, 35)} = 5.97$, $p = 0.0198$], but not day [$F_{(2, 35)} = 2.23$, NS] or an interaction between the factors [$F < 1.0$].

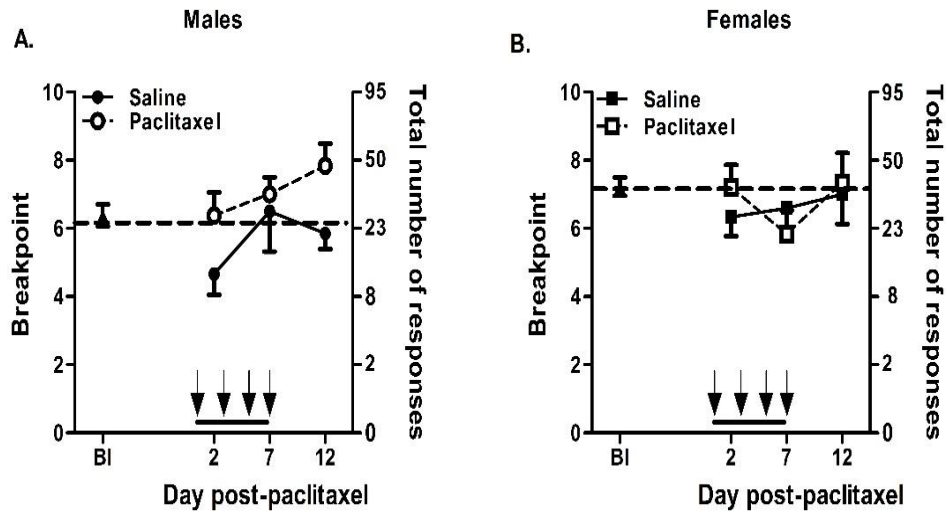


Figure 22. Morphine self-administration under a progressive ratio schedule during the development of paclitaxel-induced allodynia in male (panel A) and female (panel B) C57Bl/6 mice. Abscissa: BI (baseline prior to starting paclitaxel injections), day post first saline (filled symbols) or paclitaxel (open symbols) injection (four injections on days 1, 3, 5, and 7 are indicated by arrows). Left ordinate: Breakpoint (equivalent to the number of infusions per session); Right ordinate: Corresponding cumulative number of responses required in the PR schedule. Each data point represents mean breakpoints achieved in the 4 h session + SEM ($n = 6-15$ /group). A two-way ANOVA revealed a significant main effect of treatment ($P < 0.05$) in male but not in female mice.

In contrast, in the female group, paclitaxel failed to produce an effect on the responding for morphine under the PR schedule (Figure 22, panel B). Both saline and paclitaxel-treated groups of female mice revealed equivalent motivation to respond for morphine and a two-way ANOVA revealed no effect of treatment, day, or an interaction [$F < 1.0$].

Table 6. Paclitaxel-induced mechanical allodynia and corresponding breakpoints for morphine responding under a PR schedule during the development of chronic peripheral neuropathy in male and female C57Bl/6 mice.

Day Pre/post PAC	Mechanical Allodynia Paw withdrawal threshold		Self-administration (PR responding)	
	Males	Females	Males (0.1 mg/kg/inf) Breakpoint	Females (0.03 mg/kg/inf) Breakpoint
Bl	2.025 ± 0.25	1.5 ± 0.1	6.4 ± 0.32	7.2 ± 0.27
2	0.43 ± 0.15*	0.68 ± 0.094*	6.4 ± 0.69	7.2 ± 0.66
7	0.41 ± 0.101*	0.5 ± 0.038*	7.0 ± 0.5	5.8 ± 0.7
12	0.55 ± 0.1*	0.45 ± 0.054*	7.8 ± 0.65	7.3 ± 0.8

*, p < 0.0001 compared to baseline measure (Bl)

Figure 23, shows the effect of paclitaxel treatment on the morphine dose-effect curve established with a progressive ratio schedule. The dose-effect curve obtained with increasing unit doses of morphine in the control (saline-treated) groups of male and female mice did not demonstrate dose-dependency. Paclitaxel treatment produced a significant upward shift in the dose-effect curve for morphine in male mice (Figure 23, panel A). A two-way ANOVA revealed a significant main effect of treatment (paclitaxel) [$F_{(1, 34)} = 4.21, p = 0.048$], but not a significant effect of dose or an interaction [$F < 1.0$]. While there was an apparent upward shift in the dose-effect curve for morphine in the paclitaxel-treated female group (Figure 23, panel B), no statistically significant effect of treatment [$F_{(1, 30)} = 1.13, NS$] dose, or a treatment X dose interaction was observed [$F < 1.0$].

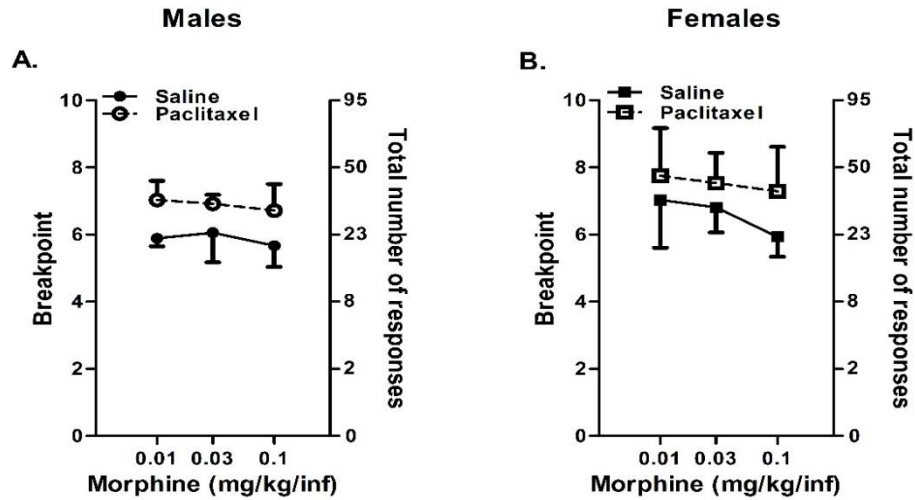


Figure 23. Effects of paclitaxel-induced chronic peripheral neuropathy on morphine dose-effect curves under a progressive ratio schedule in male (panel A) and female (panel B) C57Bl/6 mice. Abscissa: Unit doses of morphine expressed as mg/kg/inf. Left ordinate: Breakpoint (equivalent to the number of infusions per session); Right ordinate: Corresponding cumulative number of responses in the PR schedule. Each data point represents mean breakpoints achieved in the 4 h session in saline (filled symbols) and paclitaxel (open symbols) groups + SEM (n = 6-15/group). A two-way ANOVA revealed a significant main effect of treatment ($p < 0.05$) in male but not in female mice.

Figure 24 displays the percentage of subjects in the saline- and paclitaxel-treated male and female groups demonstrating an increase in PR responding for morphine from baseline (pre-injection) to post-treatment (saline or paclitaxel injections on days 1, 3, 5, and 7) test conditions at their respective training doses (males - 0.1 mg/kg/inf; females - 0.03 mg/kg/inf). In males, 57% of the paclitaxel-treated mice demonstrated an increase in breakpoints for morphine, while no mouse in the saline-treated group showed any increase in breakpoints for morphine (Figure 24, panel A). In contrast, an increase in motivation to respond for morphine under the PR schedule was observed in both the saline- and paclitaxel-treated groups of female mice (Figure 24, panel B). This

corresponded to 43% of mice in the saline-treated and 50% of mice in the paclitaxel-treated groups.

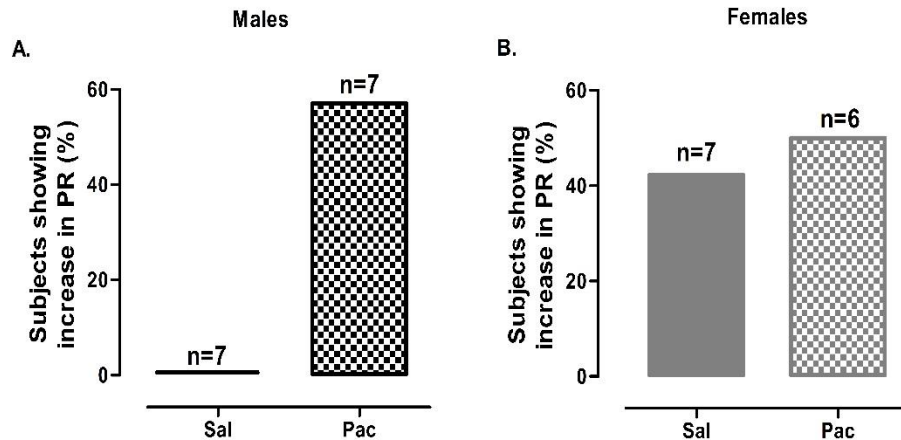


Figure 24. Percentage of saline and paclitaxel-treated male (panel A) and female mice (panel B) demonstrating an increase in breakpoints for morphine responding under a PR schedule. Abscissa: Treatment groups (Sal- saline; Pac- paclitaxel). Ordinate: percentage of subjects showing an increase in breakpoint for morphine compared to their respective baseline breakpoints. Each bar represents the values as a percentage out of $n = 6-7/\text{group}$ as indicated.

Figure 25 shows examples of response patterns from representative male mice in the PR responding for morphine at their training dose of 0.1 mg/kg/inf at baseline and post-treatment (saline or paclitaxel on day 1, 3, 5, and 7) test sessions. Initially, male mice demonstrated similar response patterns for morphine under the PR schedule at baseline. In that, mice on an average reached breakpoints corresponding to 3 to 4 infusions in the first 1 h and earned on average another 2 infusions of morphine with consistent inter-infusion intervals during the remaining 3 h of the PR session (Figure 25, panels A and C).

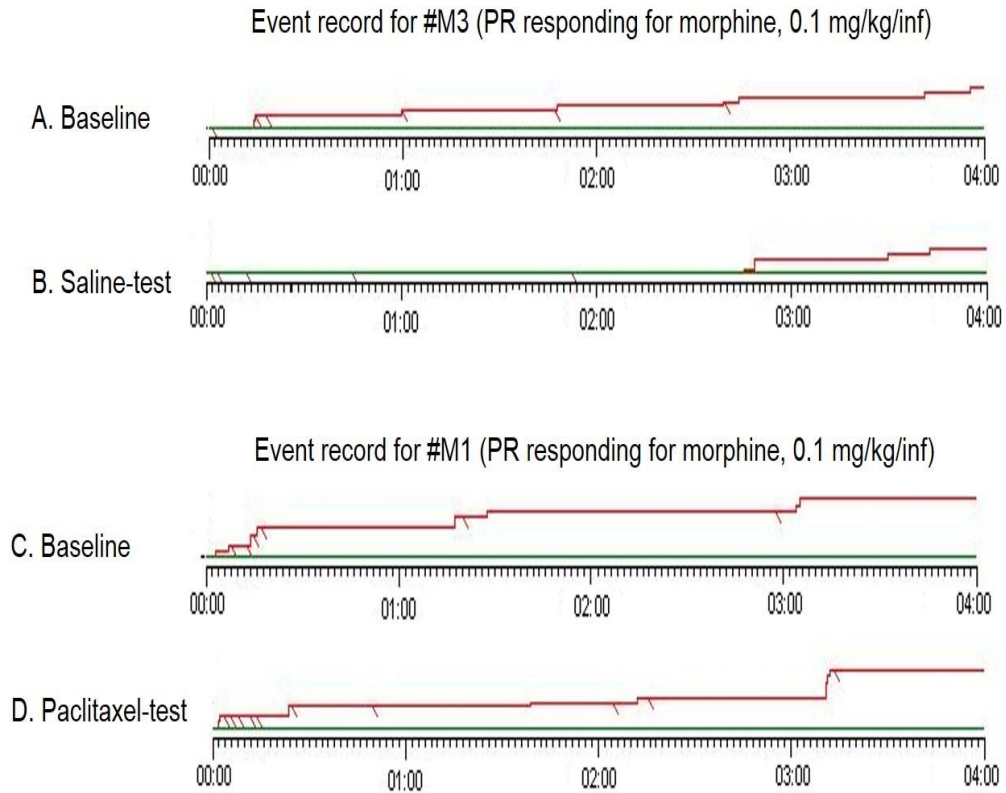


Figure 25. Representative event records at baseline (panels A and C) and post-treatment (panels B(saline) and D (paclitaxel)) for male subjects #M3 (panels A and B) and #M1 (panels C and D) responding for morphine (0.1 mg/kg/inf) under a PR schedule. In all panels, time in hours is shown along the x-axis. Morphine infusions obtained upon completion of increasing final ratios in the PR schedule are indicated by a vertical tick mark along the response scale.

Following saline treatment, the pattern of responding for morphine at this dose (0.1 mg/kg/inf) remained similar to baseline patterns (see representative patterns in Figure 25, comparing panels A and B). However following paclitaxel treatment, in a state of chronic neuropathy, the response pattern for the same dose of morphine appeared significantly altered (Figure 25, panel D). Particularly, mice responded earlier by increasing breakpoints in the first 1 h of the session and achieved higher total breakpoints

during the complete 4 h sessions compared to their own baseline pattern of responding and to saline-treated subjects' pattern of responding for morphine.

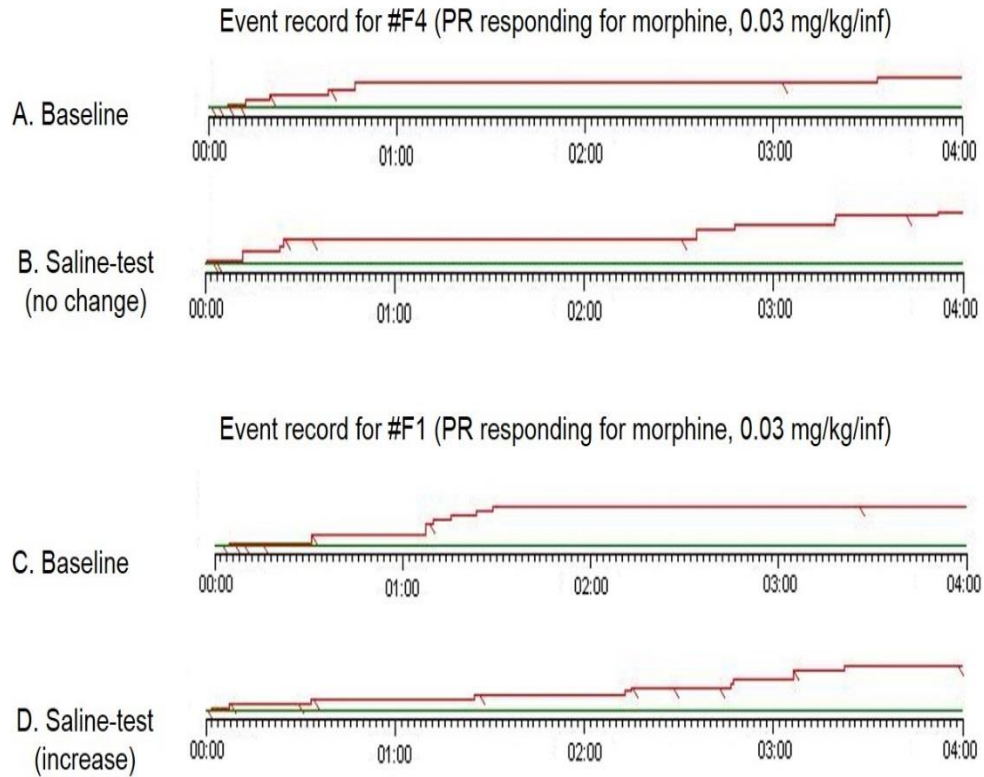


Figure 26. Representative event records at baseline (panels A and C) and post-saline treatment for female subjects demonstrating either no-change #F4 (panels A and B) or an increase #F1 (panels C and D) in responding for morphine (0.03 mg/kg/inf) under a PR schedule. In all panels, time in hours is shown along the x-axis. Morphine infusions obtained upon completion of increasing final ratios in the PR schedule are indicated by a vertical tick mark along the response scale.

Figures 26 and 27 and display examples of response patterns from representative female mice in the PR responding for morphine at their training dose of 0.03 mg/kg/inf at baseline and post-treatment (saline or paclitaxel on day 1, 3, 5, and 7) test sessions. In comparison to the response patterns for male mice, differential response patterns were observed following treatment with saline (Figure 26, panels B and D) and paclitaxel (Figure 27, panels B and D) in female mice.

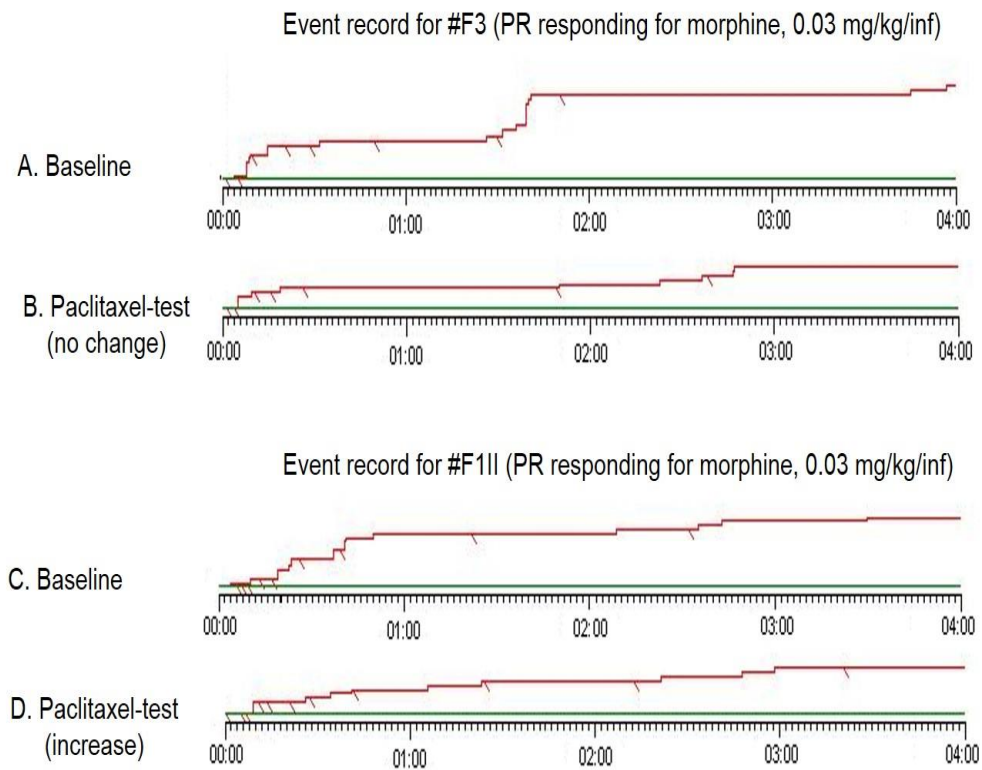


Figure 27. Representative event records at baseline (panels A and C) and post-paclitaxel treatment for female subjects demonstrating either no-change #F3 (panels A and B) or an increase #F1II (panels C and D) in responding for morphine (0.03 mg/kg/inf) under a PR schedule. In all panels, time in hours is shown along the x-axis. Morphine infusions obtained upon completion of increasing final ratios in the PR schedule are indicated by a vertical tick mark along the response scale.

Initially, while female mice displayed similar response patterns at baseline (see representative patterns in Figures 26 and 27, panels A and C), both saline and paclitaxel treatment in female mice either produced no change for breakpoints or increased total breakpoints for morphine (see representative differential patterns within saline and paclitaxel groups, Figures 26 and 27, panels B and D) compared to their baseline behaviors. The female mice that displayed an increase in breakpoints for morphine following saline or paclitaxel treatment, however, demonstrated increased responding toward the mid to end of the 4 h session (see between 2 h and 4 h in Figures 26 and 27, panel D) in contrast to a selective earlier change of increased breakpoints observed in the first 1 h of the session in male paclitaxel-treated mice.



Figure 28. Morphine intake during the first hour (panel A) and the total 4 h of the PR session (panel B) in male mice. Ordinate: Total drug intake in the 1 h (panel A) and total 4 h of PR sessions (panel B) expressed as mg/kg. Each bar represents the mean total intake achieved within the first (panel A) and the total 4 h (panel B) in saline and paclitaxel-treated male mice at baseline (BI, white bars) and post-treatment test (Test, black bars) conditions + SEM (n = 3-4/group). *, p < 0.05, compared between saline and paclitaxel test; ^, p < 0.05, compared between baseline and test within the paclitaxel group.

Figures 28 and 29 illustrate drug intake (expressed at mg/kg) in the first hour (panels A) and total drug intake in the 4 h period of the PR sessions (panels B) (calculated from the corresponding event records of mice) at baseline and post-treatment test conditions in the saline- and paclitaxel-treated groups of male (Figure 28) and female mice (Figure 29). A significant difference in drug intake was observed between the saline- and paclitaxel-treated male mice during the first hour of the PR session as determined by an unpaired t-test ($p = 0.0469$) (Figure 28, panel A). Paclitaxel-treated male mice self-administered a significantly greater amount of morphine in the presence of chronic pain compared to their own baseline (no-pain) ($p = 0.0175$) and to the amount self-administered by the saline-treated control group ($p = 0.0106$) (Figure 28, panel B). No differences were observed in morphine intake during the first hour of the PR session in both within and between saline- and paclitaxel-treated female mice. However, a statistically significant increase was noted in the total amount of morphine self-administered during the PR session in female mice from pre- to post-saline test session ($p = 0.0049$) (Figure 29, panel B).

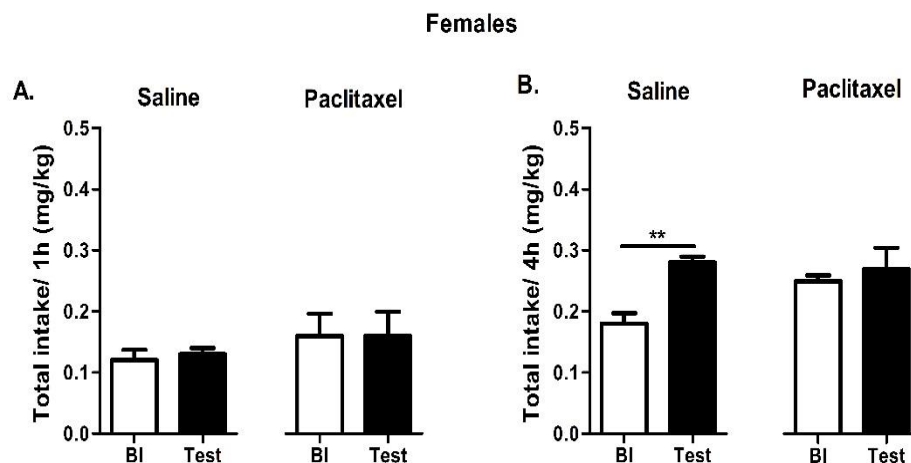


Figure 29. Morphine intake during the first hour (panel A) and the total 4 h of the PR session (panel B) in female mice. Ordinate: Total drug intake in the first h (panel A) and total 4 h of PR sessions (panel B) expressed as mg/kg. Each bar represents the mean total intake achieved within the first (panel A) and the total 4 h (panel B) in saline and paclitaxel-treated female mice at baseline (BI, white bars) and post-treatment test (Test, black bars) conditions + SEM (n = 3-4/group). **, p < 0.01, compared between baseline and test within the saline group.

Figure 30 displays the responding for saline during extinction conditions under a PR schedule in male (panel A) and female (panel B) mice. Saline-treated male mice, and saline- and paclitaxel-treated female mice displayed a trend toward extinction of PR responding when morphine was replaced by saline infusions during the 10 d repeated extinction sessions. However, in the paclitaxel-treated male mice, extinction behavior was prolonged and not observed until the end of the extinction period (10 days). A two-way ANOVA revealed a significant effect of treatment (paclitaxel) in male mice [$F_{(1, 22)} = 5.98, p = 0.0229$], but not a significant effect of day or an interaction [$F < 1.0$].

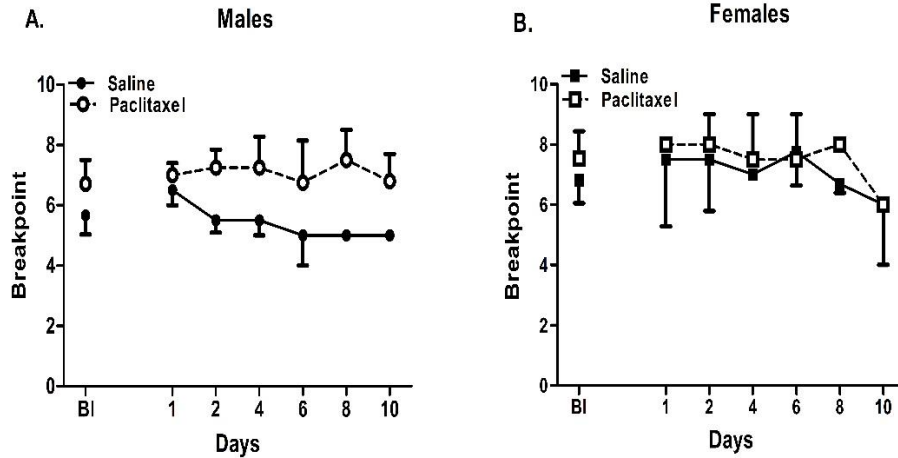


Figure 30. Ten day extinction test with only saline available under PR schedule in saline- and paclitaxel-treated male (panel A) and female (panel B) mice. Ordinate: Breakpoint (equivalent to the number of infusions per session). Each data point represents mean breakpoints achieved in the 4 h session in male [saline (filled circles) and paclitaxel (open circles)] and female mice [saline (filled squares) and paclitaxel (open squares)] + SEM ($n = 2-7/\text{group}$). A two-way ANOVA revealed a significant main effect of treatment ($p < 0.05$) in male but not in female mice.

Discussion

The current study findings provide evidence for paclitaxel-induced chronic pain to differentially modulate three aspects of opioid-induced addictive behaviors in C57Bl/6 mice using three different experimental paradigms. While the discriminative stimulus effects of the prescription opioids, morphine and oxycodone were unaltered in the presence of paclitaxel-induced peripheral neuropathy, paclitaxel treatment enhanced opioid-reward, opioid-reinforcement, and opioid-seeking behaviors in mice. Where tested, the results were opioid-selective (morphine only) and dependent on the dose of the drug. Further, the modulatory effects of paclitaxel-induced chronic pain on the reward-related behavioral effects of opioids were more pronounced and under some conditions selectively observed in male compared to female C57Bl/6 mice. To our knowledge,

several aspects of the current study are unique and documented for the first time: characterization of the discriminative stimulus properties of prescription opioids as a function of chronic pain; assessment of the motivational salience of a prescription opioid using a model with high predictive validity (progressive ratio schedule); and, evaluation of sex differences in these particular assays.

A number of studies provide evidence for a direct relationship between the subjective effects and discriminative stimulus measures of drugs in humans, and the discriminative cues of the same drugs assessed in animals using drug discrimination procedures (133, 134, 237, 238, 292). In an effort to characterize the subjective effects of prescription opioids as a function of chronic pain in mice, an opioid drug discrimination assay was used in the present study. The results revealed no effect of paclitaxel treatment on the discriminative stimulus effects of the two prescription opioids tested, relative to male and female saline controls, suggesting that the presence of chronic peripheral neuropathy may have a reduced capacity to modulate the stimulus properties of prescription opioids. While it has been shown that peripheral nerve injury-induced neuropathic pain produces neuroplastic and molecular alterations in the regions of the brain (293) that are also known to process the discriminative stimulus properties of opioids (e.g. VTA, PAG) (247, 251), the pathophysiological mechanisms underlying chemotherapy (paclitaxel)-induced peripheral neuropathy (e.g. greater peripheral and spinal cord level changes) are distinct and fundamentally different compared to surgery-induced peripheral neuropathic pain in animals (294, 295). Given these differences and the behavioral findings in the present study, it is conceivable that the discriminative stimulus effects of opioids may be differentially modulated in the presence of chronic

pain-induced by different modalities. Additional studies using other models of chronic neuropathic pain are needed to confirm this hypothesis.

In the present study, the overall conclusions about the ineffectiveness of paclitaxel treatment on opioid-induced stimulus effects in mice are confounded by the results obtained in the saline-treated control mice. Particularly, in addition to decreases in sensitivities to the discriminative stimulus effects of morphine and oxycodone observed in the paclitaxel-treated groups of both sexes, similar rightward shifts in the dose-effect curves were also apparent for the two opioids following saline-treatment in the control groups. Also, control male and female mice (3 mice in male group and 2 mice in female group) demonstrated a loss of full substitution when testing oxycodone and therefore the data pertaining to those animals were not included for the ED₅₀ value calculations and the statistical analyses. While these results might have been directly influenced by the experimental design that involved the withdrawal of mice from training during the standard dosing regimen (days 1, 3, 5, and 7) of paclitaxel or saline, these results were unexpected because previous studies in the rats demonstrated that repeated saline treatment with suspension of training for up to 2 weeks or more did not impact tolerance development or produce any decreases in sensitivity to the stimulus effects of opioids (296, 297). Moreover, the effectiveness of this assay in assessing the modulatory effects of an acute noxious stimulus on the discriminative stimulus effects of opioids were demonstrated in chapter 3 while in contrast the usefulness of this assay in the chronic pain study appears limited. These differences seen across species and across studies, however, are more difficult to reconcile at this point as currently there are no other preclinical mouse operant opioid drug discrimination studies. The current data should

provide a reference for continued studies to elucidate the importance of such variables as the duration of training, conditioning, experimental and behavioral contingencies in controlling the stimulus/drug cues, reliability of behavioral responses, and overall robustness of the opioid discrimination assay in mice.

The positive conditioned rewarding effects of morphine and oxycodone were assessed in the presence and absence of paclitaxel-induced peripheral neuropathy in male and female mice using the conditioned place preference (CPP) paradigm. Sensitivities to morphine-induced reward in the control saline-treated male and female groups are consistent with previous studies using similar doses in mice (257, 258, 298, 299). However, there are only a few, limited studies providing evidence for oxycodone-induced place preference in rodents. While the effective dose for the conditioned rewarding effects of systemically administered oxycodone in male rodents has been reported to be in the range of 0.1-1 mg/kg (300-305), the 0.3 mg/kg dose of oxycodone was without effect in C57Bl/6 mice in the current study. Specifically, while the study by Niikura et al. (305) also report a significant rewarding effect for oxycodone at the 0.3 mg/kg dose in C57Bl/6 male mice, variations in the CPP methodological parameters, including pretreatment times (30 min compared to 15 min in the present study) and number of conditioning sessions (4 pairings compared to 3 pairings of saline and drug conditioning in the present study) preclude direct comparisons with the results of that study. Although sex differences in morphine-induced conditioned reward have been reported previously in rats (76, 235) and as noted in chapter 3, the present study expanded the sex difference assessment to oxycodone-reward in mice. Saline-treated female mice revealed equivalent sensitivities to oxycodone reward relative to male mice, with no sex differences observed

in the dose-effect function (10 mg/kg producing significant CPP) and magnitude of drug reward. However, a complete dose-response effect is required to strengthen the current finding of an absence of sex differences in the conditioned rewarding effects of oxycodone in mice.

The present study revealed that the presence of paclitaxel-induced chronic pain neither enhanced nor eliminated the conditioned rewarding effects of prescription opioids, morphine (higher doses- 2.5 and 10 mg/kg) and oxycodone (10 mg/kg) relative to saline-treated control male and female mice suggesting that the susceptibility to opioid reward remains unaltered as a function of paclitaxel-induced chronic pain. The original hypothesis that paclitaxel-induced chronic peripheral neuropathy will attenuate opioid-induced reward at the higher doses tested was not supported in either sex and contrasts with the reports in male rats using a persistent inflammatory pain model (285), and in male rats and mice using a neuropathic pain model (nerve injury-induced) (113, 277, 299, 306, 307). Previous studies mentioned above reveal opioid-induced conditioned place preference (at higher doses) to be attenuated in the presence of chronic pain in male rats, likely via different mechanisms than in the present study. For example, the presence of persistent inflammatory pain has been demonstrated to up regulate kappa-opioid receptors in the nucleus accumbens of the central reward circuitry (308, 309), while the presence of peripheral nerve injury (chronic neuropathic pain) down regulates and suppresses the functioning of μ opioid receptors in the VTA (276, 277, 306), thereby reducing opioid-induced activation of the mesolimbic dopaminergic system. In contrast, administration of chemotherapeutic agents like paclitaxel are known to produce only modest degeneration and toxicity of peripheral afferent neurons and spinal microglial

hypertrophies compared to those evoked by nerve injuries in animals (295, 310) and the supraspinal pathophysiological alterations induced by paclitaxel to higher brain structures are still largely unknown. Therefore, a plausible notion is that the presence of paclitaxel-induced chronic pain is less likely to modulate the key neural substrates directly mediating opioid reward (e.g. VTA, nucleus accumbens (44, 45).

Primary findings in the present study are that the sensitivity to morphine-induced reward in paclitaxel-treated mice was augmented at the dose (0.3 mg/kg) that did not produce a CPP in the control saline-treated mice. This effect was selectively observed with morphine but not oxycodone, and the effect was more pronounced in male mice. These results are in agreement with previous reports of dose-selective alterations in morphine place-preference behavior indicating that, in rats, across different chronic pain manipulations (e.g. peripheral nerve injury-induced neuropathic pain, spinal cord injury-induced pain, inflammatory pain), the presence of chronic pain enhanced the motivation for morphine-induced reward (116, 282, 284). Cahill et al. (116) observed increased place preference following peripheral nerve injury but only at lower doses of morphine (1 and 2 mg/kg) that also effectively reversed mechanical allodynia in the neuropathic rats. These authors interpreted the increased preference for the morphine-paired context observed at these doses to be mediated by the pain-alleviating effects of morphine. Similarly in the current study, morphine-induced CPP was augmented at much lower doses than that typically used for CPP (126); that is at the 0.3 mg/kg dose in the paclitaxel-treated males an upward shift was observed, and at the 0.3 and 2.5 mg/kg doses in paclitaxel-treated female mice a leftward and upward shift was observed. However, in contrast to Cahill et al. (116), in the present study, the doses of morphine

that induced CPP in the paclitaxel-treated mice were several fold lower (males – 8-fold; females – 17-fold) than the doses that were shown to effectively reverse paclitaxel-induced mechanical allodynia in male and female mice (effective dose within the dose range tested in males – 2.5 mg/kg and females – 5 mg/kg, data shown in chapter 2).

These findings add to the growing body of literature that demonstrate nonevoked (on-going or referred to as “spontaneous pain”) components of chronic pain in animals using operant-based conditioning paradigms: CPP and conditioned place aversion (CPA) (302, 311-314) that are based on the premise that pain relief is rewarding (defined as negative reinforcement) to animals in chronic pain. Similar to the previous studies using CPA model of affective pain in rats (311, 312, 315), morphine in the present study displayed dissociable potencies to alleviate mechanical allodynia (sensory dimension) and produce CPP (nonevoked, perhaps affective/ongoing pain) in the paclitaxel-treated male and female mice. The differential potencies displayed by morphine in altering the two distinct behavioral endpoints (allodynia *versus* CPP) suggest differential mechanism that may underlie those effects, similar to the reports of the previous findings using other pain models (315-317), and consistent with clinical reports demonstrating increased effectiveness of morphine in alleviating affective rather than sensory aspects of pain (318, 319). Although the doses of morphine that produced significant CPP in the paclitaxel-treated male and female mice were the same (0.3 mg/kg), the magnitude of morphine-induced reward, relative to saline-control was greater among the male mice suggesting that male mice are more sensitive to morphine CPP (negative reinforcing effects) in the presence of paclitaxel-induced peripheral neuropathy. Moreover, the direction of this sex difference ($M > F$) is similar to what was observed for morphine’s

anti-allodynic effects (alteration of the sensory dimension) assessed using the von-Frey filament assay (chapter 2).

Another surprising finding in the present study is that, in contrast to the selective and potent effects of morphine, paclitaxel treatment failed to alter the sensitivity of mice to oxycodone-induced CPP in either sexes. Perhaps this pharmacological effect is potentially μ -selective and as pointed out in the previous chapters 2 and 3, differential opioid receptor affinity and selectivity of these two agonists (morphine - predominantly mu-receptors *versus* oxycodone – 5 to 40 times lower affinity for mu receptors; mixed kappa/mu activity) (154, 155, 208) and/or distinct pharmacokinetic profiles (201, 252, 320) for the two agonists may explain some of the observed differences. Extending these findings to other potent and high affinity μ -selective agonists (e.g. methadone, fentanyl) will strengthen this hypothesis and validate the use of this preclinical model to assess non stimulus-evoked components of chronic pain induced by paclitaxel treatment.

The conceptualization of the use of a drug self-administration model to assess drug-taking behavior in the context of a developing chronic pain state in animals has been examined (321). However, fewer studies have taken this approach preclinically in rodents to examine self-administration and chronic pain. Converging evidence is available supporting the clinical implication that the presence of untreated or undertreated chronic pain primarily maintains opioid self-administration (117-119). However, whether or not the positive reinforcing effects (reward and motivational value) of opioids are altered as a function of pain is not clear when FR schedules of reinforcement are used because rates of responding under FR schedules are influenced by the direct motor-impairing effects of opioids (130). The PR schedule of reinforcement provides a more

reliable model to predict the reinforcing efficacy and incentive-motivational properties of drugs of abuse (127, 130). The primary dependent variable in a PR schedule is the breakpoint, which is used to define the maximum effort the animal will exhibit during a given session to earn infusions of the drug, and is indicative of drug craving, drug seeking behaviors, and motivational-salience of drugs (127). The present study established that the reinforcing efficacy and the incentive-motivational value of morphine were selectively altered as a function of paclitaxel-induced chronic pain in male relative to female mice.

Male and female C57Bl/6 mice exhibited comparable morphine self-administration behaviors with no gender differences in the rate of acquisition and amount of morphine self-administered (corrected for body weight differences between sexes) under the FR1 schedule. The dose-response curve for morphine (FR1) revealed sex-specific peak effective doses (maximum response rates) for male mice (0.1 mg/kg/inf) *versus* female mice (0.03 mg/kg/inf). These findings in male mice reflect the overall consistency in the magnitude of dose-effect functions as reported widely in the literature (291, 322). However, while the observed effects in female mice (peak response rate at 0.03 mg/kg/inf) are in agreement with the limited number of studies in female rats under similar dose conditions (323), it is surprising to note that to date, morphine intravenous self-administration in female mice has not been studied. The apparent sex-specific increased response rates observed in female mice at a half log unit dose lower than that for males suggest that females may be more sensitive to the reinforcing effects of morphine similar to previous finding in rats (77). However, while previous studies have also shown female rats to acquire intravenous opioid self-administration faster (morphine and heroin) (77, 79, 324) and consume greater amounts of opioids intravenously (77, 80),

the current findings do not support those differences suggesting that perhaps the magnitude of sex-dependent differential effects of opioids may be less consistent across species (325, 326). However, additional studies are needed to resolve these differences. In the present research, male and female mice were given access to morphine under PR schedules of reinforcement prior to starting paclitaxel administration and to minimize potential interactions between the developing pain state and learning of PR responding. This is a common procedural approach used to assess effects of pharmacological or behavioral manipulations on the motivational properties of drugs (327-329). Although statistically significant sex differences were not noted at baseline, the PR responding for morphine revealed a trend toward increased breakpoints maintained in female (0.03 mg/kg/inf) compared to male mice (0.1 mg/kg/inf), which is in agreement with previous reports in the literature for morphine in rats (77, 330).

The principal finding in the present study is that in male paclitaxel-treated mice, morphine maintained higher breakpoints during the development of allodynia, which was reflected as an upward shift in the dose-effect curve. However, the shallow (flat) dose-response curve for morphine makes it difficult to assess the impact of pain on drug self-administration (shallow dose-effect curves are often observed for opioids under this PR schedule – [see discussion (128, 130, 331, 332)]). Nevertheless, the fact that the experiment was designed for potential comparisons both within and between groups gives rise to several important implications. The fact that none of the saline-treated male mice showed an increase in breakpoints for morphine while more than half of the paclitaxel-treated group showed an increase in breakpoints suggests that the observed effects are selectively mediated by the presence of the pain state and not due to

extraneous factors such as handling, injections, and/or associated stress. Although it is likely that tolerance may develop to the reinforcing effects of morphine following daily self-administration sessions over a 5 week-period (333), no tolerance was observed in the control saline group, which consistent with other reports in the literature (334).

Interestingly, the event records from the self-administration sessions revealed marked differences in the pattern of responding for morphine in the state of chronic pain in males, relative to their baseline response patterns and their saline-treated counterparts.

Specifically, paclitaxel-treated male mice reached higher breakpoints during the first hour of the 4 h self-administration session suggesting that they were displaying increased drug-seeking behavior, increased motivation to earn a greater number of morphine infusions, and were loading-up on a greater amount of the opioid early on during the session. This shift in behavioral pattern was not observed in the control saline-group.

Finally, although it is known that extinction of a learned drug-reinforced self-administration behavior is easier to achieve under an FR rather than PR schedules (335, 336), a trend to extinction responding was observed in the saline-treated control group while the extinction pattern of responding was significantly prolonged in the paclitaxel-treated male mice. This resistance to extinction was (may be suggesting increased drug craving (127)) perhaps due to stronger learned associations between the contextual-stimuli or cues and the incentive properties of the drug in the presence of a chronic pain state. Overall, these findings support the contention that the reinforcing effects and motivational-salience of morphine are augmented in paclitaxel-treated male mice relative to saline-treated controls.

In comparison to males, differential effects were observed in female mice in that an increase in responding to morphine was revealed in half of both the saline- and paclitaxel-treated groups, suggesting that the reinforcing effects of morphine increased regardless of the presence or absence of the pain state. The breakpoints maintained by morphine were not altered relative to baseline during the development of allodynia, and the upward shift in the dose-effect curve in the paclitaxel-treated group was only modest and not statistically significant. No specific changes were observed in the pattern of responding for morphine except that mice in both the saline- and paclitaxel-treated groups occasioned higher breakpoints and earned greater morphine infusions during the latter half of the drug self-administration sessions. It was previously observed in our laboratory that saline or paclitaxel administration in the current regimen, as in the present study, does not impact PR responding for food in female mice (data not shown). Therefore, the increased levels of conditioned reinforcement seen in the female mice suggest a progressive increase in sensitivity to drug-induced reinforcing effects and are consistent with a previous report where female rats were shown to consume greater amounts of heroin following long-term periods of self-administration (324). Further, these findings support the general observation in humans that females relative to males display faster progression to addiction and dependence following the initial use of opioids (see (326, 337) for reviews).

Two possible interpretations arise from the current data in male mice. First, a valid explanation of an increased propensity to opioid seeking behavior selectively observed in the context of paclitaxel-induced chronic peripheral neuropathy is that the incentive-motivational aspect of morphine was increased primarily because of the ability

of morphine to alleviate the negative subjective state and/or sensory component (e.g. allodynia) associated with paclitaxel treatment. The results are consistent with the CPP data of the present study where a similar directional increase in place preference was revealed for morphine at the low dose (0.3 mg/kg). Alternatively, since the conditioned rewarding effects of morphine at the higher doses using the CPP procedure in the paclitaxel-treated animals were similar in magnitude to the control pain-free mice, the increased PR responding for morphine may be interpreted as paclitaxel-induced pain enhancing the positive reinforcing efficacy and potentially the addiction liability of morphine. While the former interpretation is in agreement with previous studies where the presence of chronic pain in rats maintained intravenous self-administration (under an FR schedule) of prescription opioids consistent with the effectiveness of these compounds in reversing the mechanical hypersensitivity (117, 119), the latter interpretation of the present findings opposes other studies demonstrating a decrease in the positive reinforcing effects of opioids under the FR schedule (117, 119, 284, 288). The main discordance between studies is the use of different schedules of reinforcement (FR *versus* PR) that are believed to model different aspects of drug reinforcement (e.g. FR- qualitative nature of the reinforcing drug *versus* PR- quantitative relative reinforcing efficacy of drugs), differentially sensitive to experimental manipulations (130, 338), and perhaps mediated by different underlying processes and neural systems (339, 340).

Mounting evidence support the potential role of distinct brain regions such as the anterior cingulate cortex (ACC) and amygdala (central amygdala) in mediating one or more of the complex dimensions, including sensory, affective/emotional, cognitive, and motivational aspects of chronic pain (115, 316, 341, 342). Further, reward and

reinforcement behaviors mediated by drugs of abuse engage the mesolimbic neural circuits, and this circuitry together with the above-mentioned brain regions are known to modulate both pain and reward (24). It is well-established that these brain regions densely express mu-opioid receptors (343, 344) and are sexually dimorphic with respect to the binding and functional activation by the mu-opioid receptor agonists (345, 346). Therefore, future studies directed toward understanding the neurobiology of paclitaxel-induced insult in relation to the synaptic plasticity in these higher brain regions are needed to fully understand the sex-specific mechanisms underlying paclitaxel-induced modulation of opioid reward-related behaviors.

Taken together, the concurrent assessment of different aspects of addiction-related behavioral effects of prescription opioids in the same study indicated that the presence of paclitaxel-induced chronic peripheral neuropathy differentially modulates each of these behaviors, is further dependent on sex, and is selective with regard to the type and the dose of opioid tested. Continued studies focusing on the factors mediating these differential effects, combined with the growing knowledge of the multi-faceted components of chronic pain in humans, can strengthen these findings and other preclinical observations. The increased conditioned reward, reinforcing effects, and motivational aspect of morphine (at specific dose conditions) as a function of paclitaxel-induced chronic pain in males relative to a general increase in sensitivity to the rewarding/reinforcing effects of morphine regardless of pain state in females, highlights the usefulness of CPP and more importantly, i.v. self-administration using PR schedules of reinforcement as valuable preclinical tools to study the sex-specific interactions between pain-related neurobiological manipulations and opioid-induced reward. These

findings may be further amplified by human clinical and laboratory studies clarifying complex interactions between biological and psychosocial factors in the gender-dependent relationship between pain and propensity to abuse prescription opioids

CHAPTER 5

CONCLUSION

Summary of Major Findings

The current project incorporated two chemical-induced pain models to address an important and clinically relevant question: do pain states modulate the rewarding effects of prescription opioids and does this modulation vary depending on the sex of the organism? The experimental findings via systematic assessments as described in chapter 2 revealed the conditions under which the two chemicals (acetic acid and paclitaxel) produced comparable putative acute and chronic pain states in male and female C57Bl/6 mice. Importantly, administration of an intermediate concentration of acetic acid (0.4% v/v, IP) in mice did not produce any significant weight loss, motor disability, or peritonitis (upon repeated administration). Paclitaxel administration (8 mg/kg, IP) did not produce any significant weight loss, motor impairments, observable signs of spontaneous pain, discomfort, or autotomy. Moreover, both acute and chronic pain states presented with stable and consistent contextual time frames (acetic acid: acute 40 min nociceptive state; paclitaxel: 6-8 weeks persistent long-term allodynic state) during which behavioral assessments were conducted in an effort to characterize the addiction-related properties of two commonly used prescription opioids, morphine and oxycodone, as a function of acute *versus* chronic pain in male and female mice.

The results from studies in chapter 2 provided evidence for assay-specific sex differences in nociceptive and allodynic behaviors (F > M), and opioid-induced antinociceptive (F > M) and anti-allodynic effects (M > F) in C57Bl/6 mice, expanding

the current preclinical sex difference literature on pain sensitivity and opioid analgesia (see (52) for review). It was surprising to note how little information pertaining to oxycodone's effects in animal models of pain and/or addiction is in the preclinical literature, and the present data , specifically relating to the antinociceptive effects of oxycodone in the acetic acid pain model, discriminative stimulus effects and conditioned rewarding effects of oxycodone, both alone and as a function of pain in male and female mice have provided a basis for continued preclinical study of this opioid.

Few studies exist in the preclinical literature addressing a similar question on how the presence of pain alters opioid reward/reinforcement. The majority of the studies that do exist have been limited to only male rodents and a single model of chronic pain (surgery-induced neuropathic pain), although they have used different models of reward, including CPP (113, 116, 276), intracranial self-stimulation (287, 289), and oral and i.v. drug self-administration (fixed ratio schedule only) (117). The present study findings are novel in that they extend this literature to both sexes using an acute acetic-acid model and a chronic paclitaxel-induced model of neuropathic pain, involving assessments of the discriminative stimulus properties of opioids (a model of subjective effects of opioids in humans) and opioid self-administration under a progressive ratio schedule of reinforcement, as well as in both sexes. Table 7 summarizes the overall findings of the studies described in chapter 3 and 4.

Table 7. Summary of findings from the acute and chronic pain studies

Behavioral Endpoint	Dependent Variable	Acute pain		Chronic pain	
		Males	Females	Males	Females
Drug discrimination	% drug responding	↓* ^(a)	= ^(b)	↓ (non-selective) ^(d)	↓ (non-selective) ^(e)
	Response rate (morphine)	= ^(a)	= ^(b)	= ^(d)	= ^(e)
	% drug responding	= ^(a)	= ^(b)	↓ (non-selective) ^(d)	= ^(e)
	Response rate (oxycodone)	= ^(a)	= ^(b)	= ^(d)	= ^(e)
CPP	Morphine 0.3 mg/kg	= ^(c)	= ^(c)	↑* ^(f)	* ^(f)
	2.5&10mg/kg	= ^(c)	= ^(c)	= ^(f)	= ^(f)
	Oxycodone 0.3 mg/kg	X	X	= ^(g)	= ^(g)
	10 mg/kg				
Self-administration	Breakpoints (BPs)	X	X	↑* ^(h, i)	↑ (non-selective) ^(h, i)
	% mice showing increased BPs	X	X	↑ ^(j)	↑ (non-selective) ^(j)
	Response pattern	X	X	Selectively Altered ^(k)	Non-specific Change ^(l,m)
	Amount consumed (mg/kg)	X	X	↑* ⁽ⁿ⁾	= ^(o)
	Extinction	X	X	Prolonged* ^(p)	Yes ^(p)

“↑” or “↓”: selective increase or decrease in the presence of pain, *, P < 0.05
 “↑” or “↓” (non-selective): increase or decrease observed in both the pain and control groups; “X”: groups not tested; “=”: no change in the presence of pain.
 * : significant effect of treatment (posthoc tests not statistically significant)
 “Yes”: Extinction behavior was observed;
 “a-n”: Figures – 12, 14, 16, 17, 18, 19, 20, 22, 23, 24, 25(D), 26(D), 27(D), 28, 29, 30, respectively.

Taken together, acute pain induced by acetic acid selectively attenuated the discriminative stimulus effects of morphine but not its conditioned rewarding effects, whereas the opposite modulatory effect was observed for the chronic pain manipulation (paclitaxel –induced), in that the presence of chronic pain enhanced the sensitivity of mice to the rewarding and reinforcing effects of morphine, but did not alter the discriminative stimulus effects of opioids. Additionally, the differential modulatory effects of acute *versus* chronic pain were dependent on the dose and type of opioid tested (effects observed only for morphine), and as hypothesized, was more pronounced and selectively observed in male mice compared to female mice. As data were compared to appropriate control groups (water-treatment in the acetic acid experiments and saline-treatment in the paclitaxel experiments) and/or baseline behaviors of mice prior to inducing the pain states within each sex, it suggests that the effects observed were mediated by the pain-induced behavioral manipulations. Overall, these findings are consistent with anecdotal suggestions that acute pain may be less likely than chronic pain to alter opioid reward/reinforcement, and in support of human functional magnetic resonance imaging (fMRI) studies displaying more extensive engagement of brain reward circuitry components (prefrontal/limbic networks) in chronic than in acute pain-states (347). Data pertaining to parallel assessments in both sexes further provide empirical evidence that supports the implication that the reward-related behavioral effects of opioids as a function of pain will be dependent on the sex of the species.

The primary finding in the acute pain studies was that the acetic acid-induced acute noxious state selectively and sex-specifically (only in males) attenuated the discriminative stimulus effects of morphine (under specific (low) dose conditions).

These results were correlated with the data as shown in chapter 2, that under identical dose conditions, morphine was more effective in reversing the acetic acid-induced noxious state in females compared to males (therefore the modulating noxious state was more intense for males), and oxycodone was more potent in reversing the acid-induced stretches in both sexes, relative to morphine. This suggests that the degree of modulation of the discriminative stimulus effects of opioids by an acute noxious state may be inversely related to the intrinsic antinociceptive efficacy of opioids, which is further dependent on sex. The sex difference may be directly attributed (at least in part) to inherent differences in the sensitivities of male and female mice to the relative antinociceptive efficacy of different prescription opioids. These results are consistent with human laboratory studies suggesting that the subjective effects of opioids are attenuated in the presence of a painful stimuli (109, 110), although the modulatory effects are dependent on the type of opioid tested (full μ agonist *versus* mixed action agonist) (73, 236) and vary across males and females (236). The second major finding in chapter 3 was that the conditioned rewarding effects of morphine were unaltered in the presence of acute pain in both sexes, relative to saline-treated mice. Overall, the implication of these studies may be that the presence of acute visceral nociception does not impact processing of opioid effects mediated by the brain reward circuitry (e.g. VTA) (44, 45), but instead alters the brain areas, involved in regulating the discriminative cues of opioids (e.g. PAG, parabrachial nucleus) (247, 251, 255). This is further supported by preclinical studies showing these brain areas such as the parabrachial nucleus (PBn) and PAG, to be differentially engaged by visceral noxious stimuli in male and female rodents (215, 253,

254), and consistent with the clinical sex-related differences in the central processing of visceral pain in humans (199, 348).

Several critical findings collectively were obtained from the chronic pain studies in chapter 4. In contrast to the modulatory effects of acute nociception, paclitaxel treatment in mice did not alter the discriminative stimulus effects of the prescription opioids compared to the saline-treated control mice, suggesting that chronic pain manipulations may not alter the areas of the brain underlying processing of the discriminative cues of opioids (e.g., PBn, see above), perhaps governed by fundamental differences in the neurobiology of acute *versus* chronic pain (9, 15, 347). However, a potential confound from this operational assay may have been a general loss of stimulus control in mice after being removed from training in the chronic pain experiments which limits the overall conclusions. The conditioned place preference and self-administration experiments revealed two important and clinically relevant aspects of chronic pain manipulation via paclitaxel treatment in mice: i) the effects of paclitaxel-induced chronic pain on opioid-reward and the positive reinforcing efficacy of morphine; and ii) the motivational-salience of the prescription opioid as a function of chronic pain purely based on the virtue of the drug to produce pain-alleviating effects (negative reinforcement).

The first finding is that the higher doses of morphine and oxycodone were rewarding in the presence of paclitaxel-induced chronic pain in male mice, similar to the magnitude of reward observed in the control saline-treated mice. Consistently, paclitaxel treatment resulted in an increase in the maximal effort exhibited by male mice to receive morphine infusions (higher breakpoints), an increase in the consumption of the opioid and prolonged extinction responding, overall suggesting increased opioid-seeking in the

presence of an untreated chronic pain state relative to their saline-treated, pain-free counterparts. The present data support the prediction that the presence of untreated chronic neuropathic pain can increase the likelihood of opioid-seeking behavior (117) and these findings are consistent with retrospective reports and other sources suggesting the increased likelihood of aberrant drug-related and prescription opioid seeking behaviors among a subset of chronic pain patients (96, 349). In regard to sex differences in this study, paclitaxel-treated female mice demonstrated equivalent sensitivity to the rewarding effects of morphine and oxycodone compared to control mice, and a modest percentage of mice in both the saline- and paclitaxel-treated groups revealed increased breakpoints for morphine responding under a PR schedule. In addition, previous findings from our laboratory showed saline or paclitaxel treatment to not impact PR responding for food (unpublished data) suggesting that the changes observed were selective increases in sensitivity to drug reinforcement in female mice regardless of the presence or absence of chronic pain. These data support the gender-based clinical profiles of women displaying increased opioid craving (350) and accelerated trajectory from opioid use to dependence than men (326, 351), and provide preclinical empirical evidence for potential sex differences in the reinforcing effects of morphine as a function of chronic pain in mice.

The other major and important finding in the chronic pain study pertains to the growing preclinical literature exploring the affective-motivational dimension of pain via operant based animal models, one of the most bothersome and difficult aspect of clinical chronic pain to treat (23). While it is generally difficult to evaluate the affective components of pain in non-verbal animals, recent research demonstrates the benefit of

using models such as CPP and drug self-administration that are based on the premise that “pain-relief” is rewarding and reinforcing to animals in a state of chronic pain (see (24, 352) for reviews). The present study demonstrated that paclitaxel treatment enhanced the sensitivity of mice to morphine-induced reward (at a low dose of 0.3 mg/kg), and male mice were more sensitive to this effect compared to female mice. Critically important is that the same dose of morphine did not produce CPP in the control saline-treated mice, confirming that the major dissociable variable contributing to this change was the presence of an untreated chronic pain state. Further, it was intriguing to note that the paclitaxel treatment failed to alter the sensitivity of mice to oxycodone-induced CPP, relative to the potent changes observed with morphine. With the mechanism underlying the altered CPP behavior in the presence of paclitaxel-induced peripheral neuropathy still largely unknown, it is difficult to understand these disparities. Nevertheless, these data provide a reference for continued research to delineate potential factors, such as the differential pharmacological and pharmacokinetic profiles associated with the two opioids (154, 208, 252), as discussed in the previous chapters.

In line with the CPP data, morphine maintained breakpoints under a PR schedule of reinforcement that increased progressively with the time-course of allodynia development in the paclitaxel-treated male mice. In addition, more than half of the male mice in the paclitaxel-treated group revealed an increased responding for morphine under the PR schedule, and increased drug-seeking behavior. In addition, mice earned a greater number of morphine infusions, and loaded-up and titrated higher amounts of the opioid, revealing a differential drug response pattern (time-dependent shifts: increased intake of drug in the 1 h of the session). These changes were not observed in the saline-control

group. Altered morphine-reward and morphine-induced reinforcement as a function of chronic pain (negative reinforcement) were only observed in male mice at lower dose range (0.3 to 1.0 mg/kg). While the direction of sex differences (M > F) in morphine's efficacy to reverse paclitaxel-induced allodynia *versus* to produce CPP or maintain self-administration was the same, the fact that morphine was more potent in modulating reward/reinforcement behaviors compared to allodynia suggest two things: 1) the two assays are measuring different aspects of pain (e.g. CPP/self-administration: non stimulus-evoked pain *versus* allodynia: stimulus-evoked pain); and, 2) different mechanisms may underlie the two behavioral endpoints. These findings are in agreement with preclinical studies that have shown morphine to be more potent in modulating affective rather than evoked-pain using other models of neuropathic and inflammatory pain in rats (311, 315, 316, 353), and that selective mechanisms (e.g. supraspinal central/lateral amygdala-mediated) may regulate these differential effects of morphine (315, 353). More importantly, these findings are consistent with clinical reports that have demonstrated increased potency of morphine to attenuate affective rather than sensory aspects of pain (318, 319). Collectively, these data suggest that the incentive-motivational salience of morphine is increased in an untreated chronic pain state and underscores the notion that alleviation of the negative subjective state is the primary motivational drive for animals in chronic pain to display preference for an environment previously paired with an opioid (indirect drug-seeking assessment) and/or self-administer an opioid. Given that spontaneous on-going pain is a prominent and complex aspect of clinical chronic pain (21, 354), the findings support the growing literature concerning the use of operant-based animal models as preclinical tools to evaluate the

motivational aspects of pain, and more importantly, the mechanistic investigation of neural systems underlying affective components of pain that may advance development of sex-specific mechanism-based pain therapies.

Limitations

The broad aspect of the current project involved the concurrent characterization of three well established addiction-related behavioral effects of opioids as a function of pain in mice. However, some important methodological limitations associated with these approaches need to be acknowledged:

- i) One limitation of the acetic acid studies was that the opioids were administered via IP route, a less commonly used route of administration for opioids in this assay. Opioids, especially morphine, are known to have reduced bioavailability due to hepatic first pass metabolism when administered IP. Further morphine injected IP is slightly less potent than morphine injected subcutaneously in many behavioral tests making morphine slightly different from other opioids (e.g. oxycodone, methadone) that are less likely affected by first pass effect [e.g. antinociception (210), discriminative stimulus effects (355)]. However, the current studies revealed significant effectiveness for both morphine and oxycodone administered IP in the acetic acid test, and on the effects of acute pain on the discriminative stimulus effects of the opioids suggesting that both opioids are working to produce antinociception in this model. Future studies are required to verify these findings using an alternative and

commonly used subcutaneous route of opioid administration to determine if this factor contributed to the any of the sex differences and/or differences observed in the potencies for the two different opioids. In addition, it is conceivable that morphine or oxycodone could interact with acetic acid within the peritoneal cavity to reduce the number of stretches or magnitude of the noxious state. If this were the case, factors outside the central nervous system may also have contributed to the relationship between acute noxious pain state, sex, and opioids. This would further align the acute and chronic pain studies in that both stimuli (acetic acid and paclitaxel) would be peripherally administered chemical stimuli, and either or both opioids may produce antinociception or antiallodynia through peripheral opioid receptors. Additional experiments would be required to investigate this possibility.

- ii) The CPP assay is highly subject to variability in procedural manipulations across laboratories. We conducted several parametric studies based on multiple designs reviewed by Bardo et al. (246) to ensure that the assay produced robust morphine-induced CPP in C57Bl/6 mice. A reproducible assay was developed (as described in chapter 3), but a modest white-side bias was observed, so control groups conditioned with vehicle injections on both sides of the CPP compartments were included in all experiments, and the comparisons for the dose effects were always relative to the control groups. Despite the use of this modified procedure, small (but not significant) differences in the control group preference values observed

across experiments may have undermined the sensitivity of the data set to reveal statistical significance where a trend in treatment effects were observed. An alternative stimulation reward assay, such as the intracranial self-stimulation (ICSS) (356), can be used in future studies to accurately measure the ability of opioids to activate reward circuitry as a function of pain in mice (287).

- iii) The opioid drug discrimination assay adapted from a previous study in rats (237) and established for the first time preclinically in mice was effectively used to address our hypotheses in the acute pain studies. However, the conclusions based on this assay in the chronic pain study, i.e., no effect of paclitaxel treatment on the discriminative stimulus effects of opioids, were limited by a potential confound. Although stringent training criteria were followed and the experiment was designed (following pilot testing) to address the study-related questions, unanticipated differences were noted in mice (potential loss of stimulus control to opioid cues following saline treatment) that previously have not been reported to occur in control saline-treated rats under similar experimental conditions (296, 297). Future studies are needed to evaluate important variables such as the duration of training in relation to the reliability of behavioral responses in mice to better understand issues related to inherent species-specific sensitivity to drug cues.
- iv) A major limitation of the PR schedule used in the present study (130) was that it was not sensitive enough to generate monotonically increasing

dose-dependent response curves for varying unit doses of the opioid (morphine) (see (128, 331)), thereby making it inadequate for assessing the effect of pain on the unit dose effect of morphine. While otherwise the design of the study and assessment of other variables (e.g. breakpoint measures during development of allodynia, change in response patterns from baseline behaviors to post-treatment, amount of drug consumed, extinction responding) revealed several important findings, further studies are needed using a modified PR schedule where the response increments are more gradual and sensitive to support self-administration behavior maintained by a range of opioid doses (331, 332, 357). These may help clarify and dissociate the dose-specific positive or negative reinforcing effects of opioids in the context of chronic pain (as highlighted in the major findings).

Considering that one of the primary factors investigated in this project was sex differences and that female species are relatively less represented in the literature (see (120) for review), the project entailed pilot testing for the systematic characterization of baseline behavioral assessments in female mice, including complete sex-specific dose-response analyses under the three behavioral models of addiction in naïve mice prior to conducting experiments relating to the specific aims of the current project. Given the scope of the project and the long-term nature of a number of these experiments, the current investigations were limited predominantly to testing the prescription opioid morphine, while oxycodone was selectively tested in certain experiments where significant effects were observed with morphine, in order to compare two different

opioids. Further, the studies used one model each of acute and chronic pain that limited the extent to which the key findings can be generalized across pain models, stimulus modalities, and varying intensities of pain states. Although testing of a range of other commonly used prescription opioid drugs with high abuse potential (e.g. hydrocodone, methadone, fentanyl) and the use of additional pain models would have added immense value to the current project, the current models should provide a fundamental framework for future investigations to expand these findings.

Finally, another limitation of the present studies is that testing was conducted only in adult gonadally intact female mice. In fact, it is known that hormonal cyclicality and gonadal hormone functioning can impact sensitivity to pain, opioid-induced antinociceptive and reward-related behavioral effects in rodents (see (156) for review). Therefore, the experimental variability that often emerged in the data set for control female groups across experiments may be attributed to the modulatory effects of the cycling hormones that were not controlled for in these studies. While these studies are the first step in characterizing potential sex differences to engage adult intact species, hormonal manipulation is a logical next step to investigate the role of sex hormones as a potential factor that may underlie the observed sex differences in the current studies.

Future Directions

A logical extension of continued research on sex differences will be to determine the mechanisms contributing to the observed sex-specific variations in the above studies. It has already been identified that sex hormones can play an important role in modulating sex-specific behavioral responses to pain and opioid-induced effects (162, 346, 358-361).

Specifically, gonadectomy has been shown to modulate nociception in females (increase sensitivity to noxious stimuli in the estrus phase), and decrease opioid antinociception in male and increase opioid antinociception in female rodents (170, 171). Furthermore, ovariectomized female rats with estrogen replacement display increased sensitivity to the reinforcing effects of drugs of abuse (including opioids) (81, 337). Therefore, gonadectomized mice with and without hormone replacement treatments can be incorporated in the current pain models to elucidate the effects of sex hormones on sensitivity to pain behavior, opioid-induced antinociceptive/anti-allodynic effects, opioid-induced rewarding/reinforcing effects, and subsequently the interaction between pain and opioid reward as a function of gonadal steroids.

The selective increase in the reinforcing efficacy of morphine in the presence of chronic pain (in males compared to a sensitized response in females independent of pain-state), suggests that the PR schedule of drug self-administration in the context of an untreated chronic pain state may provide a valuable and sensitive model to capture the positive and/or negative reinforcing effects of drugs. However, to strengthen and validate this model, testing of two hypotheses are imperative:

- i) First, recent evidence has emerged supporting the notion that developing chronic pain states result in neuroadaptations and re-organization of the circuitry underlying drug reward (limbic/prefrontal). These changes appear almost identical to the neurobiology underlying addiction and drug-dependence (particularly opioid- and alcohol-dependence) (see (362, 363) for detailed reviews). Therefore, additional drugs of abuse and other licit/illicit substances (mainly other prescription opioids, heroin, and

alcohol) should be evaluated in this model using a sensitive procedure assessing relevant behavioral variables related to drug seeking and drug craving (progressive ratio responding, pattern of responding, drug consumption, and extinction behaviors). This may help further strengthen and validate the model to measure the intersection between chronic pain and the abuse potential of opioids and other drugs of abuse (positive reinforcing efficacy of drugs).

- ii) Second, the hypothesis that increased sensitivity to morphine CPP and motivation to self-administer morphine is primarily driven by the pain-alleviating effects of the opioid in mice experiencing chronic pain needs to be clarified and confirmed in the present model. Previous preclinical studies have shown that non-opioid pharmacological treatments (e.g. clonidine and lidocaine (administered intrathecally)) either produce CPP or maintain self-administration on their own (313, 364), or decrease opioid self-administration behaviors in rats with neuropathic pain (117). Similarly, our laboratory has investigated the rewarding effects of potential non-opioid analgesics (e.g. cannabinoid compounds) using the current CPP model (unpublished data) and will continue to investigate the reinforcing effects of these compounds using the self-administration model. In addition, the hypothesis that morphine self-administration behavior is maintained primarily by the presence of an untreated-chronic pain can be confirmed by determining whether treated-chronic pain may reverse those effects. Overall, an understanding of the negative

reinforcing effects of opioids and/or other analgesics can help dissociate evoked from nonevoked measures of pain in the paclitaxel-induced model of neuropathy in mice, which still remains obscure and are not yet clearly established.

One common observation from longitudinal studies is that chronic pain has modest prevalence rates among children and adolescents, particularly among girls (365, 366). In addition, prescription opioid misuse, drug diversion, and opioid-related overdoses are equally prevalent among adolescents and adults (367, 368). Taken together, empirical studies such as testing the current model in adolescent male and female mice (~ 3 to 4 weeks old) should be conducted to determine the sex-dependent role of age as a factor in the predisposition to prescription opioid abuse within the context of chronic pain. This information will help with the development of appropriate interventions and support the need for educational programs to mitigate prescription drug misuse and diversion in this vulnerable patient population.

Finally, having conducted these studies in an inbred genetically viable mouse strain allows future researchers to expand these studies in evaluating the role of potential genetic factors, particularly the ones that are causally associated with the development of pain, responses to behavioral effects of opioids, and vulnerability to opioid abuse (e.g. human μ -1 receptor gene – OPRM1 and its genetic polymorphism (SNP A118G); catechol-O-methyltransferase gene polymorphisms, cytochrome P450 2D6 (see (369, 370) for reviews). Application of transgenic mouse models in the current study design may help clarify potential sex-genotype interactions and the contribution of these factors to opioid efficacy, drug metabolism, side-effects, and the propensity to abuse opioids in

the context of pain, and eventually advance the development of personalized pain therapies.

In conclusion, the present study revealed converging findings that shed light on the clinically relevant question and controversy concerning the abuse liability of prescription opioids within chronic pain settings. The current series of studies have identified preclinical conditions under which pain has the capacity to modulate opioid reward-related functions dependent on sex. With additional studies, the current approach can be substantiated as useful preclinical tools to further understand the overlapping neurobiology of pain and opioid abuse, ultimately leading to the development of novel mechanism-based sex-specific therapeutic targets with low addiction liability.

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