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Effects of Chemotherapy on Motivated Behavior and Opioid Reward in Rats

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Effects of Chemotherapy on Motivated Behavior and Opioid Reward in Rats

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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List of Abbreviations

ACC = Anterior cingulate cortex

ALL = Acute lymphoblastic leukemia

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid

ANOVA = Analysis of variance

CCI = Chronic constriction injury

CGRP = calcitonin gene-related peptide

CINP = Chemotherapy-induced neuropathic pain

CPP = Conditioned place preference

DNA = Deoxyribonucleic acid

DRG = Dorsal root ganglion

ED = Effective dose

FDA = Federal Drug Administration

FR = Fixed-ratio

GABA = gamma-aminobutyric acid

i.p. = Intraperitoneal

IASP = International Association for the Study of Pain

ICSS = Intracranial self-stimulation

IENF = Intra-epidermal nerve fiber

IFN = Interferon

IL = Interleukin

LH = Lateral hypothalamus

MCR = Maximum control rate

MFB = Medial forebrain bundle

MI = Primary motor cortex

MOR = Mu-opioid receptor

MPE = Maximum possible effect

MRI = Magnetic resonance imaging

NAc = Nucleus accumbens

NCI = National Cancer Institute

NMDA = N-methyl-D-aspartate

NSAID = Non-steroidal anti-inflammatory drug

PAG = Periaqueductal gray

PBN = Parabrachial nucleus

PBS = Phosphate-buffered saline

PFC = Prefrontal cortex

PGP 9.5 = Protein gene product 9.5

pSNL = Partial sciatic nerve ligation

PTX = Paclitaxel

RMTg = Rostromedial tegmentum

ROS = Reactive oxygen species

RVM = Rostral ventromedial medulla

s.c. = Subcutaneous

SA = Self-administration

SEM = Standard error of the mean

SI = Primary somatosensory cortex

SII = Secondary somatosensory cortex

SNL = Spinal nerve ligation

SNRI = Serotonin-norepinephrine reuptake inhibitor

TCA = Tricyclic antidepressant

TGF = Transforming growth factor

TNF = Tumor necrosis factor

TRP = Transient receptor potential

VPL = Posterolateral nucleus

VTA = Ventral tegmental area

List of Compounds

Chapter II:

Paclitaxel

Chapter III:

Paclitaxel

Vincristine

Oxaliplatin

Bortezomib

Morphine

Nortriptyline

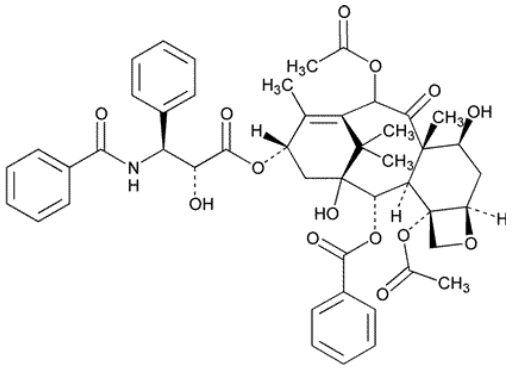
Chapter IV:

Paclitaxel

Morphine

Structure of Compounds

Chapter II:



Paclitaxel

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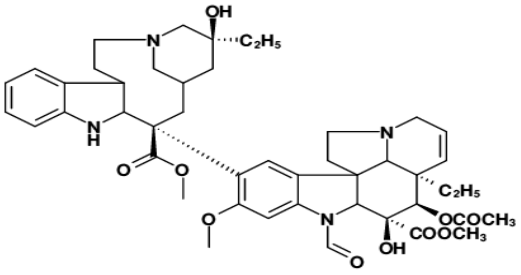
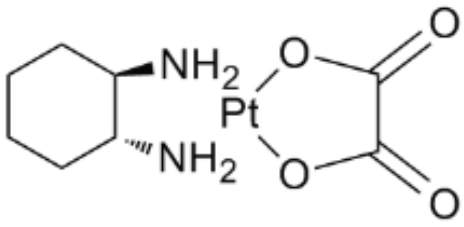
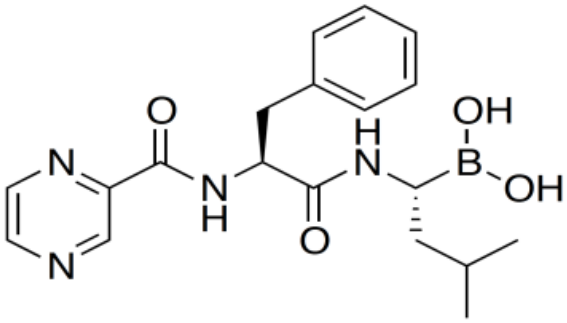


Vincristine



Oxaliplatin



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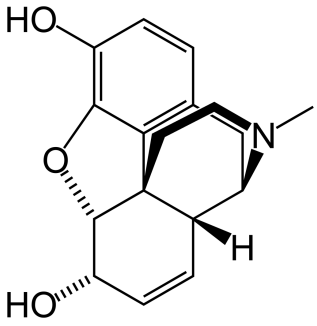
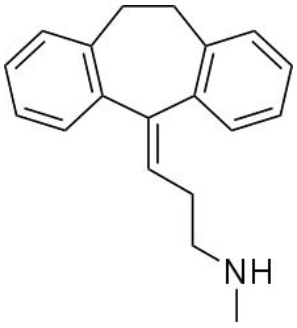


Morphine



Nortriptyline

Abstract

BEHAVIORAL EFFECTS OF CHEMOTHERAPY-INDUCED NEUROPATHY AND INTERACTIONS WITH OPIOID ABUSE

By Luke P. Legakis

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2018

Advisor: S. Stevens Negus, Ph.D.

Paclitaxel, vincristine, oxaliplatin, and bortezomib are cancer chemotherapy drugs with adverse effects that include chemotherapy-induced neuropathic pain (CINP) as well as depression of behavior and mood. In the clinical setting, opioids are often used concurrently with or following chemotherapy to treat pain related to the cancer or CINP, but repeated opioid exposure can also increase the risk of opioid abuse. This dissertation evaluated the effect of chemotherapy treatment on motivated behaviors and opioid reward in rats. The main findings of this evaluation are as follows: (1) Chemotherapy, at doses that produce robust and sustained mechanical hypersensitivity produce only weak or nonexistent depression of positively reinforced operant responding maintained either by electrical brain stimulation in an assay of intracranial self-stimulation or by food pellets in an assay of food-maintained responding. (2) There was no correlation between the expression of mechanical hypersensitivity and depression of motivated behaviors across individual animals, suggesting that these two effects of chemotherapy do not share common mechanisms of action. (3) Mechanical hypersensitivity, but not behavioral

depression could be reversed with morphine. (4) The class of chemotherapeutic used in preclinical models is a determinant of the severity of effects on neuropathy-related endpoints and on the time course of these effects. (5) Chemotherapy does not protect against the rewarding effects of repeated morphine administration and does not alter the time course of the enhancement of reward with repeated morphine exposure. These findings suggest that administration of chemotherapy to rats induces mechanical hypersensitivity while failing to decrease behaviors dependent on mesolimbic dopamine signaling or protecting against morphine abuse-related effects. While apparent that chemotherapy can produce peripheral neuropathy, the data in this dissertation does not support the hypothesis that chemotherapy can produce behavioral depressant manifestations of chemotherapy-induced neuropathic pain (CINP) in rats.

Chapter I: Introduction

1. Chemotherapy-Induced Neuropathy and Neuropathic Pain are Growing Clinical Problems with No Successful Treatments

Advancements in chemotherapy have improved the prognosis and survival rate of many forms of cancer, and chemotherapy is considered one of the great medical achievements of recent decades (Ali et al., 2013; Lambertini et al., 2017). Despite the improvements of chemotherapy compounds, which effectively kill rapidly dividing cells and enable patients to survive their battle with cancer, the side effects caused by chemotherapy can often negatively impact their quality of life for decades. One of the best examples of this medical double-edged sword is paclitaxel.

Paclitaxel is the prototype compound of the taxane class of chemotherapeutics and was discovered from a pacific yew tree in 1955 as part of a National Cancer Institute (NCI) funded plant-screening program. Its structure and synthesis was determined in 1971, and paclitaxel was finally approved by the FDA to treat ovarian cancer in 1993 (Cragg, 1998). The mechanism of action for paclitaxel's anti-cancer effect is to stabilize polymerized microtubules during metaphase, preventing the progression to anaphase in rapidly dividing cells (Schiff and Horwitz, 1980; Risinger et al., 2014) (Figure I.1). Paclitaxel is one of the most commonly administered and effective chemotherapeutics in the United States and throughout the world, and it has been used to improve survival in patients with nasopharyngeal (Miyaushiro et al., 2015), non-small cell lung (Langer et al., 2015), breast (Sparano et al., 2008), and ovarian cancers (Suh et al., 2014). Patients treated with paclitaxel often experience emesis, alopecia, and diarrhea during the treatment period, but these effects diminish with the cessation of the drug (Reeves et al., 2012). Paclitaxel also produces chemotherapy-induced peripheral neuropathy in roughly 60% of

patients (Seretny et al., 2014), with the presence of chemotherapy-induced neuropathic pain (CINP) reported in approximately half of those with peripheral neuropathy, or 30% of total patients (Lavoie Smith et al., 2011). Paclitaxel-induced peripheral neuropathy manifests clinically as somatosensory deficits such as paresthesia (abnormal sensations) or dysthesia (unpleasant abnormal sensations) that can exist in the absence or presence of concurrent CINP, and unlike the other adverse effects, peripheral neuropathy and CINP can be irreversible and impact patient well-being for decades (Golan-Vered and Pud, 2013). For example, CINP is associated with signs of functional and emotional impairment including decreases in days healthy enough to work (Pike et al., 2012), functional mobility (Davies et al., 2016; Miaskowski et al., 2017), cognitive function (Ando-Tanabe et al., 2014), and increases in fatigue, hopelessness, and depressive symptoms (Pedersen et al., 2007). The emergence of these signs can limit paclitaxel dose ranges that can be used in cancer treatment (Speck et al., 2013) (Table I.1).

Paclitaxel is not the only chemotherapeutic known to produce CINP. Vincristine, oxaliplatin and bortezomib are three other compounds used to treat cancer that also produce peripheral neuropathy and CINP. Vincristine is another plant-based compound originally extracted from the rosy periwinkle or "vinca" plant and belonging to a class of drugs known as the vinca alkaloids. The periwinkle plant has been used as a remedy for centuries, and vincristine was approved by the FDA for treatment of leukemia in 1963 following rodent and human studies demonstrating efficacy (Johnson et al., 1963). The mechanism of action for vincristine's anti-cancer effect is through arresting cells in metaphase by binding to β-tubulin and inhibiting the polymerization into microtubules (Schlaepfer, 1971; Owellen et al., 1972) (Figure I.1). Vincristine has been very effective in treating acute lymphoblastic leukemia (ALL) and

Hodgkin's lymphoma and is also often used to treat other solid tumors such as lung, breast, and squamous cell cancer of the head/neck (Crom et al., 1994; Bowman et al., 1996; Horning et al., 2002). Patients treated with vincristine often experience constipation, hyponatremia, and alopecia during the treatment period, but these effects diminish with the cessation of treatment (Bohannon et al., 1963; Nicholson and Feldman, 1972). Vincristine also can produce sustained peripheral neuropathy in roughly 20-30 percent of patients, and this is a dose-limiting effect of the drug (Ramchandren et al., 2009; Seretny et al., 2014). Higher rates of neuropathy are found when cumulative doses are > 4 mg/m^2 and in older patients (Lavoie Smith et al., 2015). Most patients receiving vincristine are children due to the typical age of onset of acute lymphoblastic leukemia and Hodgkin's lymphoma. Even minor neuropathy can have long-term consequences for childhood cancer survivors as decreased physical activity, obesity, type 2 diabetes mellitus, and cardiovascular disease have been linked to motor and sensory deficits produced by vincristine (Hoffman et al., 2013; Khan et al., 2014). The degree to which vincristine produces depression-like symptoms during or after treatment in this mostly young patient population is unknown (Table I.1).

Oxaliplatin is a platinum-based synthetic compound that was discovered by a Japanese professor, Dr. Kidani, in 1979 and was first approved by the FDA for the treatment of colorectal cancer in 2002 (Kidani et al., 1980). The anti-tumor mechanism of action for all platinum-based chemotherapies is to interfere with mitosis and cell division by crosslinking intra-strand deoxyribonucleic acid (DNA) within regions rich in guanine and cytosine nucleotides. The cross-linked DNA inhibits replication and transcription, leading to a fatal effect on cells in the synthesis phase of the cell cycle (Faivre et al., 2003) (Figure I.1). Oxaliplatin is most commonly and effectively used to treat colorectal cancer, often being administered in conjunction with 5-

fluorouracil (de Gramont et al., 2000; Wilson et al., 2002). Patients treated with oxaliplatin typically experience nausea, emesis, diarrhea, and potential nephrotoxicity (Spunt et al., 2007) as well as neurological adverse effects such as ototoxcity and/or an acute peripheral neuropathy with a robust hypersensitivity to cold stimuli followed by a more chronic sensory neuropathy in 75-90 % of patients and CINP is present in approximately 15% of patients (Cersosimo, 2005; Ramanathan et al., 2010; Seretny et al., 2014). It is the chronic sensory neuropathy that is coincident with CINP and responsible for limiting cumulative oxaliplatin dose and disrupting physical functioning (Cersosimo, 2005). Oxaliplatin treatment has been linked to decreased physical functioning (Serrano et al., 2014), increased depression, and decreased self-perception (Lynn et al., 2017) (Table I.1)

Bortezomib is the prototypical drug of the chemotherapeutic class of proteasome inhibitors and was first synthesized and developed by Myogenics/Millennium Pharmaceuticals in 1999, later receiving FDA approval for the treatment of multiple myeloma in 2008 (Teicher et al., 1999). Bortezomib's mechanism of action against cancerous plasma cells is not fully understood, but the most promising theory is that bortezomib changes the fate of the "immortal phenotype" plasma cells by allowing apoptotic and cell checkpoint-inhibitor proteins to accumulate and function (Gelman et al., 2013). In cancerous plasma cells, it is thought that the proteasome is degrading pro-apoptotic and checkpoint inhibitor proteins too quickly for them to effectively function, allowing the cell to proliferate and escape these apoptotic checks. Bortezomib, through its boron atom, binds the 26S proteasome and inhibits its function (Bonvini et al., 2007). Bortezomib has been shown to improve survival and is most efficacious in the treatment of multiple myeloma (Mikhael et al., 2009; Bringhen et al., 2010) and of mantle cell lymphoma (Fisher et al., 2006). Patients treated with bortezomib often experience nausea,

emesis, and diarrhea. Patients are also at risk for thrombocytopenia (a dose-limiting effect) in 30-50% of patients as well as deficiencies in other components of blood (Iwamoto et al., 2010). Bortezomib is also able to produce peripheral neuropathy in the form of sensory deficits in 35-55% of patients and CINP in approximately 25% of patients (Dimopoulos et al., 2011; Seretny et al., 2014; Lee et al., 2017). Bortezomib has also been linked to increased depression and severity of neuropathy compared to thalidomide for multiple myeloma (Hjorth et al., 2012). The effects of bortezomib on physical functioning are unknown (Table I.1).

Paclitaxel, vincristine, oxaliplatin, and bortezomib have improved the survival and prognosis of patients with many different forms of cancer. In fact, three of them are on the World Health Organizations (WHO) List of Essential Medicines, with the lone exception being bortezomib, which has dramatically improved the survival rates of patients with multiple myeloma, particularly in those who do not receive hematopoietic cell transplantation (Mikhael et al., 2009; Bringhen et al., 2010). While the classes of drugs discussed here (taxanes, vinca alkaloids, platinum-based anti-tumors, and proteasome inhibitors) produce the most severe and prevalent forms of peripheral neuropathy and CINP, epothilones such as ixabepilone and immunomodulatory drugs such as thalidomide are also known to produce these signs and symptoms. The advancements in the past 50-60 years in chemotherapy treatment have allowed patients diagnosed with cancer to live significantly longer and potentially cancer-free. Following this grueling battle, patients can be faced with the new challenges of side effects caused by the cures in the form of peripheral neuropathy, CINP, and/or functional impairment. At present, there are no adequate treatments to prevent or reverse chemotherapy-induced neuropathy, CINP, or pain-related functional impairment (Dworkin et al., 2010; Finnerup et al., 2015).

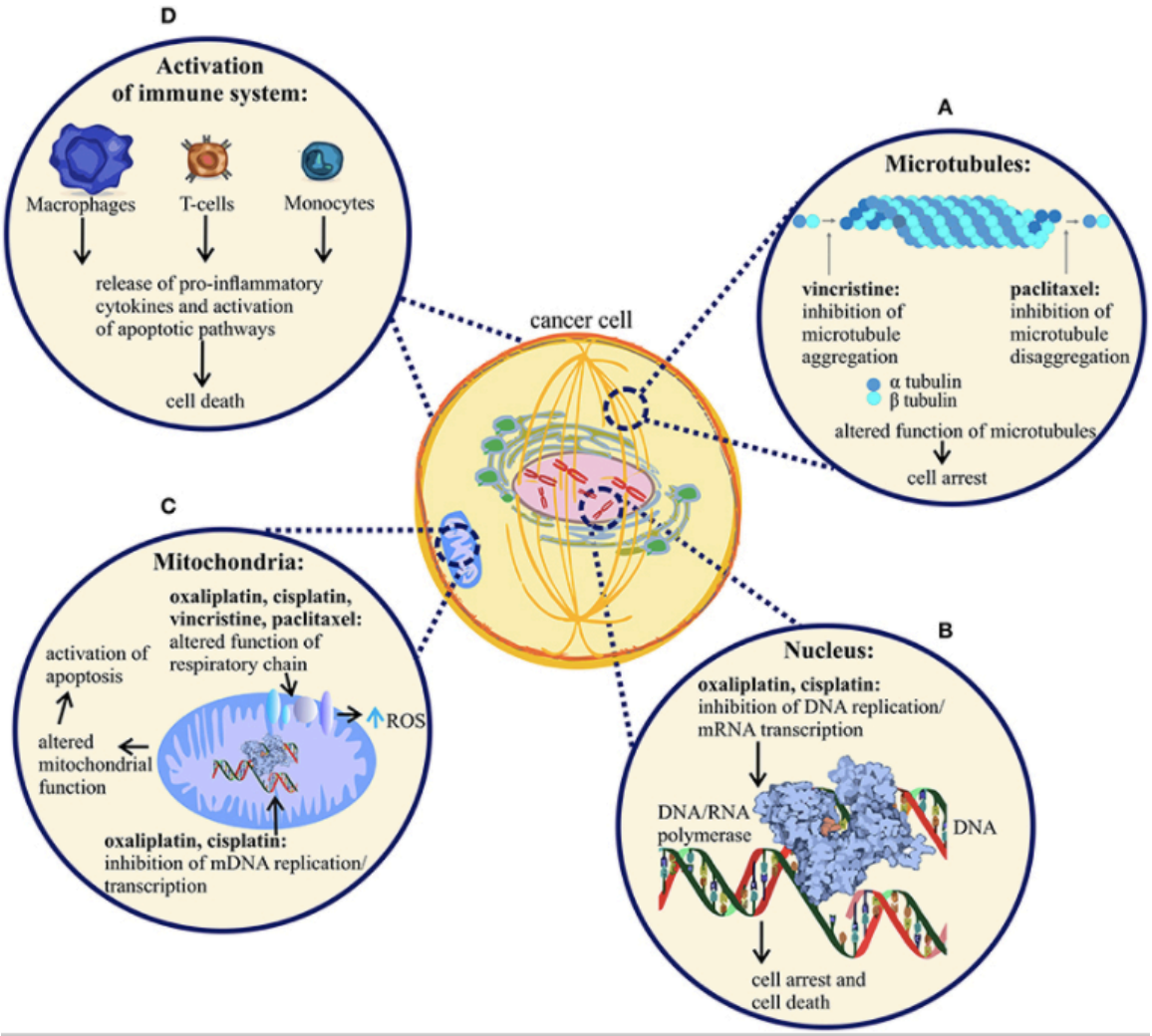


Figure I.1: Mechanism of action of paclitaxel, vincristine, oxaliplatin and cisplatin. Panels A-B display mechanism of action of paclitaxel, vincristine, oxaliplatin, and cisplatin to produce cell arrest and cell death in dividing cells. (A) Paclitaxel hyperstabilizes microtubules preventing disaggregation and vincristine prevents microtubule assembly. (B) Oxaliplatin and cisplatin bind to nuclear DNA, disrupting transcription and replication. Panels C-D display mechanism of action of paclitaxel, vincristine, oxaliplatin, and cisplatin to produce cell death in any cell, including neurons. (C) All four agents alter mitochondria function and increase the production

of reactive oxygen species (ROS). Oxaliplatin and cisplatin can cause damage by binding to mitochondrial DNA disrupting transcription and replication. (D) All four agents activate immune cells and secretion of cytokines and chemokines. Adapted from Starbova and Vetter, 2017 (Starobova and Vetter, 2017).

Table I.1: Clinical effects of paclitaxel, vincristine, oxaliplatin, and bortezomib

Drug	Paclitaxel	Vincristine	Oxaliplatin	Bortezomib
GI effects	**Nausea, emesis, diarrhea** (Reeves et al., 2012)	**Nausea, constipation** (Bohannon et al., 1963)	**Nausea, emesis, diarrhea** (Cersosimo, 2005)	**Nausea, emesis, diarrhea** (Iwamoto et al., 2010)
Incidence of peripheral neuropathy	**50 – 70 %** (Seretny et al., 2014)	**20 – 30 %** (Ramchandren et al., 2009; Seretny et al., 2014)	**75 – 90 %** (Cersosimo, 2005; Ramanathan et al., 2010; Seretny et al., 2014)	**35 – 55 %** (Dimopoulos et al., 2011; Lee et al., 2017)
Incidence of CINP	**30 %** (Lavoie Smith et al., 2011)	**No data found**	**15 %** (Ramanathan et al., 2010)	**25 %** (Dimopoulos et al., 2011)
Effect of physical function	↓ (Hoffman et al., 2013; Davies et al., 2016; Miaskowski et al., 2017)	↓ (Hoffman et al., 2013; Khan et al., 2014)	↓ (Cersosimo, 2005; Serrano et al., 2014)	**No data found**
Effect on depression or depression-associated symptoms	↑ (Pedersen et al., 2007)	**No data found**	↑ (Lynn et al., 2017)	↑ (Hjorth et al., 2012)
Other adverse effects	**Alopecia** (Reeves et al., 2012)	**Alopecia, hyponatremia** (Nicholson and Feldman, 1972)	**Nephrotoxicity, ototoxicity, cold hypersensitivity** (Cersosimo, 2005; Spunt et al., 2007)	**Thrombocytopenia** (Iwamoto et al., 2010)

N/A =No available data found for a drug on a particular endpoint

2. Mechanisms of Chemotherapy-Induced Peripheral Neuropathy, Neuropathic Pain, and Changes in Motivation.

Chemotherapy induced peripheral neuropathy causes sensory disruptions in the hands and feet in the form of hypoesthesia (decreased sensitivity to stimulation), paresthesia (an abnormal sensation, whether spontaneous or evoked), and dysthesia (an unpleasant abnormal sensation, whether spontaneous or evoked). The damage caused by these drugs is predominately in the long axons of large myelinated nerve fibers (Quasthoff and Hartung, 2002; Han and Smith, 2013), but the cause and progression of this damage is not fully understood. One of the paradoxes obstructing researchers' understanding of the mechanisms of chemotherapy-induced neuropathy is that the compounds responsible for the death of rapidly dividing cells are also damaging neurons that are not dividing and proliferating, but are in an alternate resting stage of the cell cycle commonly referred to as G_0. The mechanism of damage to nerves is likely different from the mechanism for the drugs' anticancer effect. Chemotherapy-induced neuropathy, regardless of the causal agent, is a dose- and treatment time-dependent effect with increased exposure increasing the likelihood and severity of neuropathy (Grisold et al., 2012; Seretny et al., 2014). Oxidative stress, ion conductance changes, activation of neuroinflammation, and axonal degeneration are present in most forms of chemotherapy-induced neuropathy and are some of the proposed mechanisms for the cause and progression of nerve damage (Starobova and Vetter, 2017). However, just as the different classes of chemotherapeutics act on different targets to produce a similar anti-cancer phenotype (reduced or eliminated cancer burden), there is evidence to suggest that the different classes produce nerve damage through different mechanisms to produce a similar neuropathic phenotype as outlined in Figure I.2.

Figure I.2

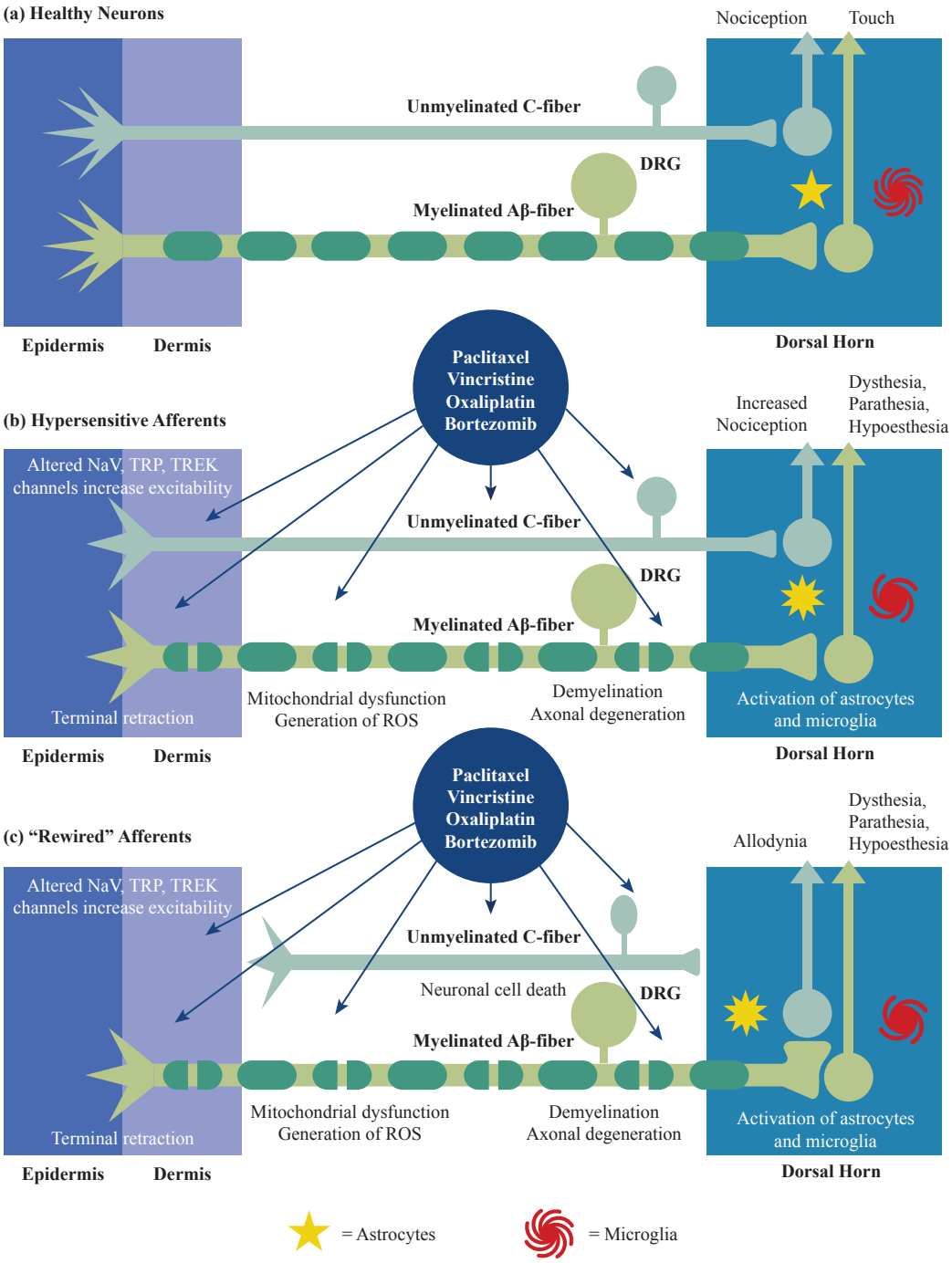

(a) Healthy Neurons
Nociception
Touch
Unmyelinated C-fiber
DRG
Myelinated Aβ-fiber
Epidermis
Dermis
Dorsal Horn
Paclitaxel
Vincristine
Oxaliplatin
Bortezomib
(b) Hypersensitive Afferents
Increased Nociception
Dysthesia, Parathesia, Hypoesthesia
Altered NaV, TRP, TREK channels increase excitability
Unmyelinated C-fiber
DRG
Myelinated Aβ-fiber
Terminal retraction
Mitochondrial dysfunction
Generation of ROS
Demyelination
Axonal degeneration
Activation of astrocytes and microglia
Epidermis
Dermis
Dorsal Horn
Paclitaxel
Vincristine
Oxaliplatin
Bortezomib
(c) "Rewired" Afferents
Allodynia
Dysthesia, Parathesia, Hypoesthesia
Altered NaV, TRP, TREK channels increase excitability
Unmyelinated C-fiber
Neuronal cell death
DRG
Myelinated Aβ-fiber
Terminal retraction
Mitochondrial dysfunction
Generation of ROS
Demyelination
Axonal degeneration
Activation of astrocytes and microglia
Epidermis
Dermis
Dorsal Horn
= Astrocytes
= Microglia

Figure I.2: Schematic indicating the multiple adverse effects of paclitaxel, vincristine, oxaliplatin, and bortezomib on primary afferent neurons and glia. Panel a displays a healthy sensory neuronal system. An unmyelinated C-fiber capable of transmitting nociceptive information and a myelinated Aβ-fiber capable of transmitting innocuous-touch information have free nerve endings or dendrites within the epidermis of skin, cell bodies residing in the dorsal root ganglion (DRG), and terminals in the dorsal horn of spinal cord. Panel b displays the adverse effects of the four chemotherapies on neurons and glia that may cause (1) increased nociceptive signaling due to hyperexitability of C-fibers, (2) dysthesia and paresthesia due to hyperexcitability of Aβ-fibers, and (3) hypoesthesia due to terminal retraction and damage to free nerve endings and dendrites in the epidermis. Panel c displays the adverse effects of the four chemotherapies on neurons and glia that may cause "rewiring" of afferent information. Hyperexcitable Aβ-fibers with decreased dendritic density in the epidermis may cause dysthesia, paresthesia, and hypoesthesia as well as induce nociception to non-noxious stimuli (allodynia) due to reorganization of circuitry in the dorsal horn whereby Aβ-fibers innervate and activate spinal dorsal horn neurons normally activated by nociceptors. Based on figure 1 of Boyette-Davis et al., 2015 (Boyette-Davis et al., 2015)

Oxidative stress, particularly within mitochondria of neurons, is one of the potential mechanisms for chemotherapy-induced neuropathy. Chemotherapy treatment has been shown to increase reactive oxygen species (ROS) in the mitochondria in cells, and these increased ROS levels can damage intracellular machinery, proteins, and molecules leading to demyelination, and both sensitization and desensitization of intracellular signaling pathways, including increasing apoptotic pathways (Sangeetha et al., 1990; Look and Musch, 1994; Weijl et al., 1998; Cashman and Hoke, 2015). There are also several drug-specific effects observed in terms of oxidative stress in the mitochondria. Paclitaxel, by modulating mitochondrial membrane permeability, can cause mitochondrial depolarization and release of calcium from mitochondria, an intracellular signal of cell damage, in both neuronal and non-neuronal cells (Kidd et al., 2002; Mironov et al., 2005). Oxaliplatin can not only bind and crosslink nuclear DNA to prevent replication and translation, but can also bind and crosslink mitochondrial DNA as well, disrupting mitochondrial functioning and causing intracellular increases in ROS (Canta et al., 2015) (Figure I.1). Additionally, oxalate, a metabolite of oxaliplatin, is an effective chelator of extracellular Ca^{2+}. The decreases in extracellular Ca^{2+} observed with oxaliplatin have been correlated with increasing neuronal sodium conductance and increasing the excitability of neurons (Deuis et al., 2013). It is often suggested that oxalate is responsible for the robust cold hypersensitivity in the acute phase of oxaliplatin-induced neuropathy as oxalate alone can produce cold hypersensitivity in animals (Deuis et al., 2013). Vincristine can alter also Ca^{2+} signaling in mitochondria, though the causes and consequences of this finding are not known (Carozzi et al., 2015). The best evidence of a causal role in neuropathy for vincristine's modulations of calcium signaling is that concurrent use of verapamil, a calcium channel blocker, results in an increased incidence of neuropathy symptoms (Kaba et al., 1985). Lastly, both vincristine and paclitaxel induce swollen

and vacuolated mitochondria in nerves (Flatters and Bennett, 2006). In all of these examples of mitochondrial disruption and oxidative stress, it is unknown if these are signs of damage, crucial steps in the pathophysiology, or the root cause of chemotherapy-induced neuropathy (Figure I.2).

In addition to modulation of both intra- and extracellular calcium signaling, at least three other ion channels have been implicated in the pathophysiology of chemotherapy-induced neuropathy: voltage-gated sodium channels, voltage-gated potassium channels, and transient receptor potential (TRP) channels. Paclitaxel can increase the incidence of spontaneous activity from 4.8% to 27.1% in large sized and from 0 to 33.3% in medium sized dorsal root ganglion neurons with a decreased rheobase, indicating enhanced excitability. Real-time polymerase chain reaction (rt-PCR) studies in these neurons indicated significant upregulation of Nav1.7 sodium channels (Zhang and Dougherty, 2014). Oxaliplatin has been shown to modulate several channels, possibly due to the chelating properties of the oxalate metabolite. In nerve conduction studies performed in patients with oxaliplatin-induced peripheral neuropathy, significant increases in relative refractory periods correlated with altered NaV channels in the nodes of Ranvier along the axon (Krishnan et al., 2005). Additionally, in recordings of human and mouse peripheral axons, exposure to oxaliplatin and cold induced action potential bursts in myelinated Aβ and Aδ (but not unmyelinated C) fibers. In dorsal root ganglion (DRG) neurons taken from Scn8a knockout mice that lack functional NaV1.6 channels, no action potential bursts were observed when exposed to oxaliplatin and cold (Sittl et al., 2012). Oxaliplatin can also produce increased neuronal excitability by downregulating the expression of TREK1 and TRAAK, potassium channels that aid in hyperpolarizing neuron axons. In TREK1 and TRAAK knockout mice, cold hypersensitivity was eliminated (Descoeur et al., 2011). A potential pathway of vincristine CINP has been proposed using mice. Following vincristine administration,

infiltration and activation of $CX3CR1^+$ monocytes by CXC3CL1 (also known as fractalkine) led to an increased production of ROS that activated $TRPA1^+$ sensory neurons, resulting in increased hypersensitive withdrawal responses (Old et al., 2014).

Although chemotherapeutics are notorious for temporary immunosuppression (leukocytes and monocytes are rapidly dividing cells) and risk of infections, they can also produce activation of certain components of the immune system. The potential role of immune cells and signals in the pathophysiology of chemotherapy-induced peripheral neuropathy relies mostly on the use of immunological suppressors to reverse chemotherapy-induced hypersensitivity of withdrawal responses to mechanical stimuli in rodents accompanied by relatively limited evidence of immunoactivation in humans attributed to chemotherapy (Lees et al., 2017). The clinical evidence for chemotherapy-induced immunoactivation includes an analysis of blood samples from non-small cell lung cancer patients treated with paclitaxel, and this analysis revealed increased production and expression of interferon (IFN)-gamma, interleukin (IL)-2, and CD44 among $CD4^+$ and $CD8^+$ T cells (Zhang et al., 2008). Another clinical study involving 24 patients with small-fiber neuropathy (most not caused by chemotherapy) found that patients with neuropathy had a 2-fold higher expression of the proinflammatory IL-2, the anti-inflammatory interleukin IL-10, and transforming growth factor (TGF) β_1 (Uceyler et al., 2010). One possible interpretation of this result is that changes observed in inflammatory modulators are a consequence of neuropathy and not a cause. It is important to note, however, that absence of clinical evidence for chemotherapy-induced activation of the immune system does not signal evidence of absence of these potential effects.

In rodents, paclitaxel increased activation of Schwann cells (Cavaletti et al., 1995), microglia, and astrocytes (Ruiz-Medina et al., 2013). Vincristine administered to rodents

increases tumor necrosis factor (TNF)-alpha mRNA and protein while activating microglia and astrocytes in the spinal dorsal horn (Kiguchi et al., 2008). Additional preclinical evidence to support the hypothesis that chemotherapy-induced neuropathy is mediated through activation of the immune system is in the form of the attenuation or exacerbation of chemotherapy-induced mechanical hypersensitivity with the administration or genetic manipulation of immune modulators following treatment with paclitaxel (Raghavendra et al., 2003; Ledeboer et al., 2007; Boyette-Davis et al., 2011a; Zhu et al., 2011), vincristine (Callizot et al., 2008), and oxaliplatin (Boyette-Davis and Dougherty, 2011; Di Cesare Mannelli et al., 2014; Makker et al., 2017).

It is likely that a combination of oxidative stress, mitochondrial dysfunction, and an activated immune system all play a role in producing axonal degeneration, a clear sign of neuropathy (Figure I.2). A case study of a patient who received a cumulative dose of paclitaxel >6600 mg (17 cycles), showed severe fiber loss, axonal atrophy, and demyelination (Sahenk et al., 1994). In 37 patients with vincristine-induced peripheral neuropathy defined as hypoesthesia and motor deficits, nerve conduction velocities decreased with the progression of treatment. Sural nerve biopsies from the three (out of 37) patients with vincristine-induced peripheral neuropathy and concurrent CINP found many fibers demonstrating myofibrillary disruption, axonal degeneration, and phagocytosis of myelin debris (Bradley et al., 1970). Rodent studies have complemented the clinical studies in this regard, demonstrating retraction of intra-epidermal nerve fibers (IENFs) out of the epidermis following paclitaxel (Cavaletti et al., 1995; Bennett et al., 2011; Boyette-Davis et al., 2011a) and oxaliplatin (Boyette-Davis and Dougherty, 2011) treatment, and this decrease in intra-epidermal nerve fibers correlates with observed chemotherapy-induced hypersensitivity of withdrawal responses from mechanical stimuli. There has been one study that has looked at all four of the major classes of chemotherapeutics that

cause peripheral neuropathy. In Boehmerle et al., 2014, paclitaxel and vincristine produced axonal degeneration primarily in large myelinated fibers while bortezomib produced axonal degeneration primarily in small myelinated fibers. The platin-based antitumor drug in this study, cisplatin, damaged both large and small myelinated fibers to a similar extent (Boehmerle et al., 2014). These findings suggest that the chemotherapeutic agent used can be a determinant of the type of fiber damaged.

Regardless of the pathophysiological mechanisms producing peripheral neuropathy, chemotherapy damage to nerves coupled with immune stimulation can lead to increased pain sensation in patients through processes outline in Figure I.2. There are largely two prevailing theories as to how neuropathy can paradoxically produce hypoesthesia, as well as increased pain and hypersensitivity to mechanical and thermal stimuli. The first (currently more popular) theory involves modifications of the primary and secondary afferent neurons, and these modifications cause inappropriate responses to stimuli. Hypoesthesia can be attributed to the dying back of primary afferent Aβ-fiber mechanoreceptor terminals out of the epidermis (Boyette-Davis et al., 2011b; Boyette-Davis et al., 2015). Pain, dysthesia, and paresthesia could be attributed to altered functioning of voltage-gated sodium channels (NaV1.7 and NaV1.6), voltage-gated potassium channels (TREK1 and TRAAK), or TRP channels (TRPA1) located on primary afferents as a result of mitochondrial stress, ROS generation, abnormal Ca^{2+} signaling dysfunction, and/or infiltration and activation of immune cells (see above, Figure I.2). Additionally, disrupted glutamate neurotransmission and/or inflammatory mediators released by astrocytes and microglia within the dorsal horn of the spinal cord could modulate synaptic signaling and post-synaptic response of secondary afferent neurons (Cata et al., 2006; Robinson and Dougherty, 2015). These changes could produce hypersensitivity and dysthesia responses to

stimuli. The second theory involves spinal cord plasticity at the synapses between primary and secondary afferents in the dorsal horn of the spinal cord. Due to the neuropathy described above, neuronal cell death of unmyelinated C-fiber nociceptors can often occur. In the newfound absence of these fibers, other primary afferents (including Aβ-fibers) may fill the void to provide new and abnormal inputs to secondary nociceptors. This "cross-wiring" may result in abnormal activation of secondary nociceptors by inputs from primary afferents initially activated by innocuous stimuli. When this transmission is sent to the brain, it will have information coded for both mechanical sensation as well as pain sensation. With the increased prevalence of primary afferent cell death, synaptic plasticity may contribute to the experiences of dysthesia, parathesia, and allodynia (pain due to a stimulus that does not normally provoke pain). The concept of spinal plasticity was first tested and demonstrated by Clifford Woolf in 1983. In decerebrate rats exposed to a unilateral thermal foot injury, the surviving neurons showed increased discharges and receptor fields within the dorsal horn of spinal cord (Woolf, 1983). These findings have been expanded to animals exposed to a neuropathy-inducing stimulus (Suzuki et al., 2000; Suzuki et al., 2005).

Peripheral neuropathy, CINP, and depression of behavior and mood represent chemotherapy's chronic adverse effects. Pain, regardless of cause (CINP or otherwise), often leads to depression of behavior, and relief from pain-related depression is a goal of analgesics (Bair et al., 2003; Dworkin et al., 2005; Dharmshaktu et al., 2012). In fact, the International Association for the Study of Pain (IASP) defines "pain" as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". This definition implies the role of both pain sensation and higher order brain functions to experience pain and to protect against further damage by decreasing behavior, a process

beneficial to human and animal survival, encoded through millions of years of evolution. This process begins by following the same neural track as nociception, the neural coding of noxious stimuli that are capable of producing tissue damage (Figure I.3). Primary afferents with receptors capable of detecting noxious stimuli are activated by those noxious stimuli and are termed primary nociceptors. These primary afferents are pseudounipolar neurons with dendrites or free nerve endings in tissue such as skin, muscle, and viscera, cell bodies in the DRG, and terminals in lamina I and II of the dorsal horn of spinal cord (ipsilateral). Typically, these primary nociceptors can be broken down into two types based on their fiber diameters, presence or absence of myelination, and conduction velocities: large myelinated Aδ-fibers with fast conducting axons and small unmyelinated C-fibers with slow conducting axons. In addition, primary nociceptors can either respond exclusively to noxious stimuli or can display graded responses spanning innocuous stimuli to noxious stimuli, termed "wide dynamic range" neurons. Activation of receptors on primary nociceptors producing suprathreshold depolarization in membrane potential triggers an action potential that propagates down the axon and results in the release of glutamate and other neurotransmitters such as substance P and calcitonin gene-related peptide (CGRP) at the terminal into the synapse of the dorsal horn. Glutamate, the primary neurotransmitter at the dorsal horn synapse, activates α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, producing excitatory currents in the secondary afferent neuron. The secondary afferent neurons, with cell bodies within the ipsilateral dorsal horn of spinal cord, have axons that decussate the spinal cord through the anterior white commissure and travel up the contralateral lateral spinothalamic tract

Figure I.3

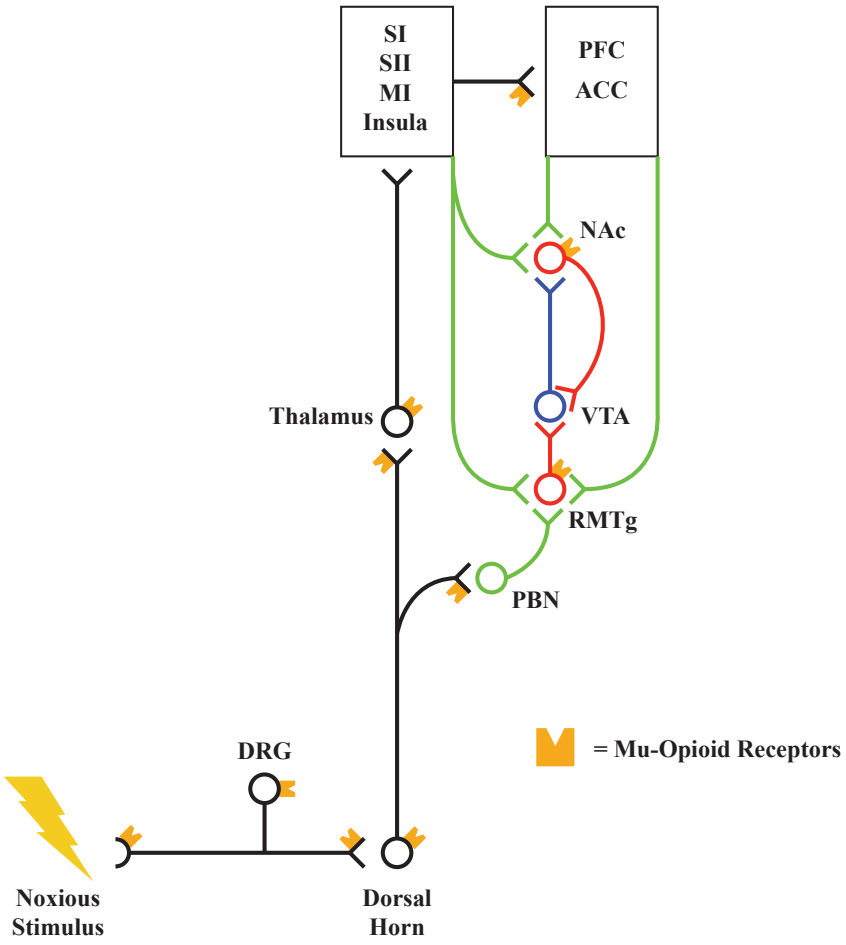


Figure I.3: Schematic outlining the trisynaptic ascending pain pathway (black neurons), the mesolimbic dopamine neurons (blue), and the "bottom-up" and "top-down" modulatory circuits by which activity in the pain pathway can modulate activity in mesolimbic dopaminergic neurons. Neurons in the modulatory circuit are green for excitatory neurons and red for inhibitory GABAergic neurons. The location of mu-opioid receptors in these circuits is also indicated. Noxious stimuli cause activation of the primary nociceptors with cell bodies within the dorsal root ganglion (DRG). Primary afferents synapse onto secondary spinothalamic tract nociceptors that have cell bodies in the spinal dorsal horn and project to the thalamus. Tertiary thalamocortical neurons transmit nociception signals from thalamus to cortical regions such as SI

(primary somatosensory cortex), SII (secondary somatosensory cortex), MI (primary motor cortex), and insula. Nociceptive information in the cortex is often then transmitted through quaternary neurons to higher-order regions such as prefrontal cortex (PFC) and anterior cingulate cortex (ACC). There are at least two proposed circuits whereby activity in this ascending nociceptive neurons can influence mesolimbic dopaminergic signaling from ventral tegmental area (VTA) to nucleus accumbens (NAc). Dopaminergic projections from VTA and NAc play a critical role in motivated behaviors and the rewarding effects of drugs of abuse. The "bottom-up" circuit involves excitatory (green) transmissions from parabrachial nucleus (PBN) receiving nociceptive information from ascending lateral spinothalamic tract neurons to GABAergic neurons in the rostromedial tegmentum (RMTg). The RMTg can send inhibitory (red) transmissions to the VTA. The "top-down" circuit involves excitatory transmissions from higher-order cortical regions such as PFC and ACC that descend to GABAergic neurons in NAc and RMTg that can inhibit dopamine neurons within the VTA. Analgesic effects of mu agonists involve binding to mu receptors on ascending nociceptive neurons to inhibit activity of those neurons. Abuse liability of mu agonists involves binding to mu receptors on GABAergic neurons in NAc and RMTg to inhibit those neurons and subsequently disinhibit mesolimbic dopamine neurons.

and project onto the tertiary neurons with cell bodies within thalamic nuclei such as the ventral posterolateral nucleus (VPL). At this synapse, glutamate once again is the primary neurotransmitter, activating the tertiary neurons through AMPA and NMDA receptors. These thalamic neurons project to cortical targets including the primary somatosensory cortex (S1), primary motor cortex (M1), anterior cingulate cortex (ACC), prefrontal cortex (PFC) and insular cortex. These regions of the brain, as well as other brain centers receiving input as part of the pain pathway (Figure I.3) including hypothalamus, amygdala and ventral tegmental area (VTA), produce both the cognitive and emotional sensation of pain.

How pain causes depression of behavior is not fully understood, but there is strong evidence that dopaminergic neurons originating in VTA and projecting to regions such as nucleus accumbens (NAc) as part what is known as the mesolimbic pathway are involved (Stellar and Stellar, 1985; Wise, 2008). Activation of dopaminergic projections from VTA to NAc is associated with the expression of motivated behavior and the reinforcing effects of stimuli that include brain stimulation, food pellets, and drugs of abuse (Di Chiara and Imperato, 1988; Wise, 1996). Moreover, inhibition of mesolimbic dopaminergic neurons is correlated with depression of behavior in acute pain states (Borsook et al., 2007; Wood, 2008; Jarcho et al., 2012; Leitl et al., 2014a). For example, intraperitoneal (i.p.) acute lactic acid in rats depressed both extracellular dopamine release in the NAc and rates of reinforcement earned in operant procedures. Clinically effective opioid and non-steroidal anti-inflammatory drugs (NSAIDs) were able to block the acid-induced depression of extracellular dopamine in NAc and operant behavior (Leitl et al., 2014a). The effects of a chronic pain state on mesolimbic dopamine signaling are relatively unknown.

There are at least two potential neural pathways by which a pain state can cause depression of behavior through inhibition of mesolimbic dopamine signaling, and they can be referred to as the "bottom-up" and "top-down" pain pathways (Figure I.3). The bottom-up pathway, as in the nociception pathway described above, involves activation of primary afferents by noxious stimuli and neurotransmission to secondary afferents in the spinal dorsal horn. Traveling up the spinal cord of the lateral spinalthalamic tract, there are collaterals to brainstem regions such as the rostral ventromedial medulla (RVM), parabrachial nucleus (PBN), periaqueductal gray (PAG), and hypothalamus (Figure I.3). Of note, the PBN within the dorsolateral pons appears to be a critical link in the neurotransmission of nociceptive information to dopaminergic neurons of the VTA. Tracer analysis of neuronal projections label anterograde connections from PBN to the substantia nigra pars compacta and the VTA of rats. Axonal terminals originating from PBN were observed adjacent to dominamergic neurons of the VTA. Extracellular recordings during the application of footshock displayed excitation of the PBN neurons and short-latency inhibition of dopaminergic neurons of the VTA (Coizet et al., 2010). This inhibitory input to the VTA from PBN is achieved through both direct and indirect gamma-aminobutyric acid- (GABA)-ergic signaling (Basbaum et al., 2009; Bushnell et al., 2013). Additionally, chemically induced visceral pain can increase transcription factors such as c-Fos in PBN, a sign of activation and increased production of proteins for neurotransmission, further promoting the idea that PBN is a key offshoot of the lateral spinothalamic tract (Lanteri-Minet et al., 1993). A possible intermediary in neurotransmission from PBN to VTA and NAc is the rostromedial tegmental nucleus (RMTg), which is populated largely by GABAergic neurons. The VTA also receive GABAergic input from the RMTg, and noxious stimuli can increase c-Fos expression in the GABAergic neurons of RMTg (Jhou et al., 2009; Barrot et al., 2012).

In addition to the "bottom-up" pathway, in the "top-down" pathway, the nociceptive signal has reached cortical regions of the brain that have inhibitory connections to the VTA. Higher-level regions of the brain including the ACC, insular cortex, and prefrontal cortex (PFC) that receive signals from the thalamus and other regions may have important direct and indirect input into the VTA and NAc. The administration of noxious stimuli produces activation of ACC (Shackman et al., 2011; Wager et al., 2013), insular cortex (Kross et al., 2011; Wager et al., 2013), and PFC (Sogabe et al., 2013; Vadovicova, 2014), all cortical components of what is occasionally referred to as the "Pain Matrix," or brain regions believed to be involved in functional networks allowing humans to perceive pain (Fomberstein et al., 2013). The ACC, activated as part of the ascending pain signal (Apkarian et al., 2005), can modulate and enhance nociceptive signaling through the rostral ventral medulla (RVM) (Calejesan et al., 2000) and direct modulation of spinal neurons (Chen et al., 2014). These regions send indirect connections back up to VTA, and in addition, proposed direct connections between ACC and VTA can decrease dopaminergic signaling to NAc. The posterior insula is the region of the brain most consistently activated in the presence of a pain state, though its role in pain processing is not fully understood. Functional MRI studies have demonstrated a predictive and correlative stimulation between the insula and VTA of human subjects when delivered a thermal noxious stimulus (Fairhurst et al., 2007). It is not clear if this relationship is direct or indirect. Imaging studies in patients with chronic back pain suggest that a strong connection between PFC and NAc exists that is not present in control patients (Baliki et al., 2010), while later studies demonstrated the presence of functional connectivity between PFC and NAc was predictive of the persistence of back pain (Baliki et al., 2012). Cortical regions that become activated as part of ascending nociception such as ACC, insula, and PFC may have "top-down" effects on both

nociception and mesolimbic dopamine signaling that could be modulating pain-depressed behavior.

3. Preclinical Assessment of CINP and Pain-Depressed Behavior

In preclinical research, administration of noxious stimuli is used induce changes in behavior that can be quantified and utilized to test candidate and known analgesics. Assays to assess nociceptive behaviors can be categorized as evaluating either pain-stimulated behaviors, defined as behaviors that increase in frequency, rate, or intensity with exposure to a noxious stimuli, or pain-depressed behaviors, defined as behaviors that decrease in frequency, rate, or intensity with exposure to noxious stimuli (Negus et al., 2006). Assays evaluating pain-stimulated behaviors are widely used, and examples include tail-flick responses to noxious thermal stimuli (Juszkiewicz-Donsbach and Levy, 1962), stretching responses induced by i.p. administration of noxious chemical stimuli (Eckhardt et al., 1958), and paw-withdrawal responses from thermal and mechanical stimuli (Reeh et al., 1986). Analgesics decrease pain-stimulated behavior by reducing sensory sensitivity to noxious stimuli, but "false positive" decreases in pain-stimulated behaviors can also be produced by drugs that sedate or impair motor function. Pain-depressed behaviors are underutilized in preclinical research and include noxious-stimulus induced depression of unconditioned behaviors such as feeding (Stevenson et al., 2006; Kwilasz and Negus, 2012), locomotion (Cobos et al., 2012), and nesting (Negus et al., 2015). Analgesics can restore pain-depressed behaviors by again decreasing sensory sensitivity to noxious stimuli, but drugs that produce motor impairment only exacerbate pain-related behavioral depression and do not produce "false positive" analgesic-like effects in assays of pain-depressed behavior. Drugs that produce non-selective stimulation of behavior can produce

"false positive" increases pain-depressed behaviors, but this type of false positive is less common because non-selective motor stimulation may increase behaviors different from the behaviors depressed by noxious stimuli. Incorporation of both pain-stimulated and pain-depressed behaviors enhance the validity and translation of potential therapeutic drugs.

Administration of chemotherapy has been used as a noxious stimulus to model to chronic pain, neuropathic pain, and CINP. Most commonly, paclitaxel is the chemotherapeutic tested, and it reliably produces hypersensitive paw-withdrawal responses from mechanical stimuli in rodents that can last for weeks and months (Polomano et al., 2001; Pascual et al., 2010; Boyette-Davis et al., 2011a; Hwang et al., 2012; Ko et al., 2014; Toma et al., 2017). Translation of paclitaxel-induced effects seen in humans has been poor for primarily two reasons. First, the presence of chemotherapy-induced neuropathy is defined clinically as sensory dysfunction and most commonly manifests as numbness and hypoesthesia of the hands and feet, indicating decreased sensitivity. CINP, defined as a patient answering "Yes" to the question of "Are you currently in pain?" when there is no exposure to additional stimuli, is seen in a smaller proportion of patients and can be considered a sign of spontaneous pain (Table I.1). Allodynia, defined as reporting pain when exposed to normally innocuous mechanical and thermal stimuli, is observed in an even smaller population still and can be considered a sign of evoked pain (Golan-Vered and Pud, 2013). Second, the behavioral assessment of allodynia in humans and rodents are very different. In human studies the presence of pain when exposed to mechanical stimuli is conveyed through a verbal report, whereas in rodent studies the nociception and inferred presence of pain when exposed to mechanical stimuli is conveyed through withdrawing a paw from the stimulus.

In rodents, numerous treatments have been identified that alleviate chemotherapy-induced mechanical allodynia, a type of pain stimulated behavior, by reducing the frequency of paw withdrawals to mechanical stimuli; however, none of these medications have proven to be reliably effective in clinical treatment of either CINP or neuropathic pain (Sindrup and Jensen, 1999; Xiao et al., 2008; Hama and Takamatsu, 2016). A large factor impeding preclinical-to-clinical translation of candidate analgesics is the type of dependent measure used to indicate the presence of "pain," and novel pain-depressed behavioral assays have been developed with the goal of modeling pain-related functional impairment and improving translation (Martin et al., 2004; Negus et al., 2006; Mogil, 2009). For example, operant responding reinforced either by electrical brain stimulation directly stimulating reward centers of the brain or by food pellets that involve a more complicated neuronal circuitry can serve as baseline behavior that can be depressed in rodents by some noxious stimuli (Martin et al., 2004; Ewan and Martin, 2014; Negus et al., 2015; Warner et al., 2015). Utilization of pain-depressed conditioned behaviors such as ICSS and operant responding for food are advantageous because data obtained address the weaknesses burdening assessment of pain-stimulated behaviors. Depressed motivated behavior and functional impairment are cardinal signs of CINP in humans, whereas allodynia is not. Additionally, candidate drugs producing motor impairment can produce analgesic-like but false-positive decreases in withdrawal responses but would fail to reverse noxious-stimulated behavioral depression (Sindrup and Jensen, 1999; Xiao et al., 2008; Hama and Takamatsu, 2016).

Intracranial self-stimulation (ICSS) is one type of baseline behavior that can be used to evaluate expression and treatment of pain-depressed behavior. In ICSS, subjects are implanted with intracranial electrodes that target specific brain regions, and operant responses result in the

consequent delivery of electrical stimulation to that target. ICSS has advanced the understanding of the neuroanatomy of reward. The discovery that dopaminergic projections from VTA to NAc are involved in reward is due in part to early experiments of ICSS where rats vigorously earned brain stimulation when electrodes were placed in particular brain regions, including those that activate mesolimbic dopamine projections (Olds and Milner, 1954). The mesolimbic dopamine pathway between VTA and NAc has become synonymous with "reward pathway". While maintaining the essence of Olds and Milner's experimental paradigm, current ICSS procedures often involve the implantation of microelectrodes into the medial forebrain bundle to activate mesolimbic dopamine signaling (Figure I.4). ICSS procedures produce reliable baseline rates of behavior that can be depressed following administration of a noxious stimulus, and pain-related ICSS depression can be blocked or reversed by clinically effective analgesics but not by non-analgesics that produce motor impairment (Martin et al., 2004; Ewan and Martin, 2014; Leitl et al., 2014a; Leitl et al., 2014b). Importantly, pain-related depression of ICSS has been correlated with pain-related depression of dopaminergic signaling within NAc following administration of acute i.p. lactic acid (Leitl et al., 2014a). Operant assays that use food pellets as positive reinforcers can also provide reliable baseline rates of behavior that can be depressed following administration of a noxious stimulus (Warner et al., 2015). Behavior reinforced with food pellets is of interest to those studying the effects of chemotherapy because a notable adverse effect of chemotherapy is marked weight loss and decreased appetite. Utility of ICSS, as well as operant responding maintained by other reinforcers such as food pellets, can be used to assess pain-depressed behavior as one component of preclinical analgesic behavioral assessment for CINP in conjunction with mechanical hypersensitivity assays.

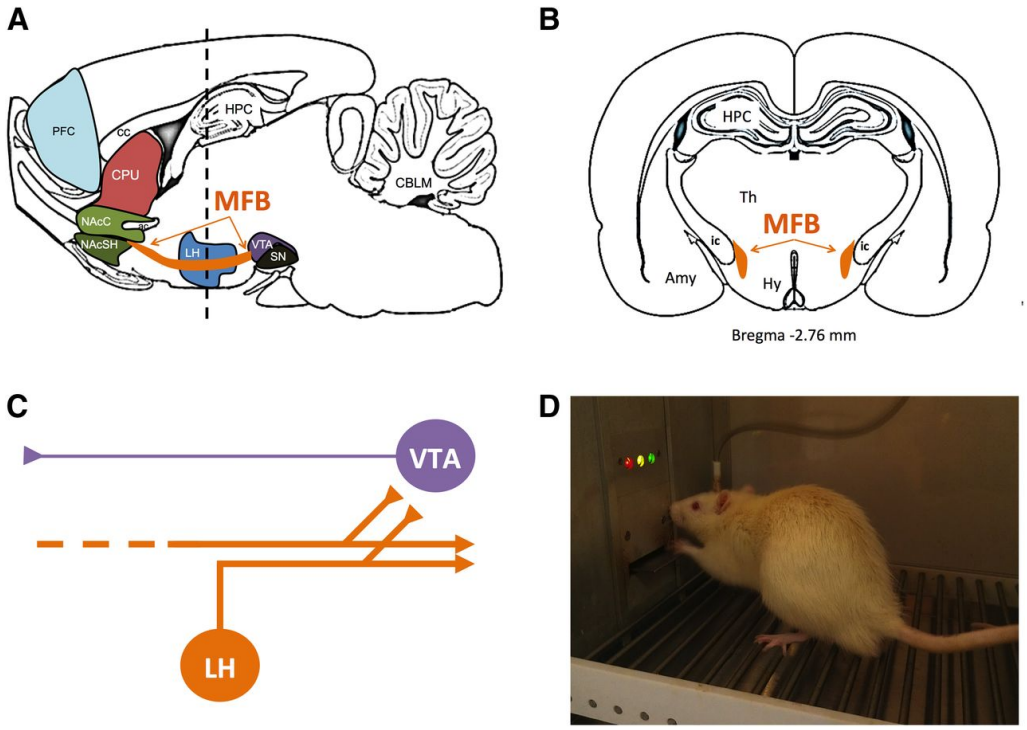


Figure I.4: The medial forebrain bundle (MFB), at the level of the lateral hypothalamus (LH), is a fiber tract that contains both ascending and descending axons, and it serves as a target region for brain stimulation in intracranial self-stimulation (ICSS) studies for assessment of motivated behaviors. (A) Sagittal section of rat brain showing MFB in orange. (B) Coronal section of rat brain showing MFB in orange. (C) Schematic of neurons thought to contribute to ICSS. Electrical stimulation via an implanted microelectrode produces direct activation of descending myelinated neurons (orange) that originate in lateral hypothalamus and project caudally to midbrain and brainstem. Collateral branches of these neurons project to and activate unmyelinated dopamine neurons (purple) in ventral tegmental area (VTA). (D) Photograph of a rat with a MFB planted electrode in an operant chamber. Amy, amygdala; CBLM, cerebellum; cc, corpus calloscum; CPU, caudate/putamen; ic, internal capsule; HPC, hippocampus; Hy, hypothalamus; NAcC, nucleus accumbens core; NAcSH, nucleus accumbens shell; PFC, prefrontal cortex; SN, substantia nigra; Th, thalamus.

4. Opioids and Chemotherapy-Induced Neuropathic Pain; Analgesia and Abuse

Opioid analgesics and the poppy plant are among the oldest known drugs in human history and can be traced back to one of the earliest historical documents with the first written record of opioid drugs written by the ancient Greek poet Hesiod in eighth century B.C. (Kritikos and Papadaki, 2001). The efficacy of opioid analgesics to treat most pain states, and the importance and utility of these drugs as medical tools, should not be underplayed (Kalso et al., 2003; Canovas-Martinez et al., 2015; Kopecky et al., 2017). However, throughout history, the poppy plant has not only been a symbol and a source of relief from painful calamities, but also of death in the form of toxic overdose. The Greek god of death, Thanatos, was often depicted with a wreath of poppies around his neck. The rise of prescription opioid analgesics for the treatment of pain has enabled opioid abuse and overdose deaths to reach epidemic proportions in the United States.

Opiate addiction is characterized by use of opioid analgesics to the detriment of the user's physical, psychological, and social health. Dependence and tolerance, while often present in addiction, are not necessary for the diagnosis of opioid use disorder (American Psychiatric Association, 2013). Initial opioid exposure can occur during medically supervised treatment for acute or chronic pain, but data published in the 1980s were interpreted to suggest that risk of addiction was low under these conditions (Porter and Jick, 1980; Portenoy and Foley, 1986). This perception likely contributed to the dramatic escalation in clinical opioid use that occurred through the 1990s and up to the present (Sehgal et al., 2012; Wilson-Poe and Moron, 2017). However, more recent evidence suggests that rates of iatrogenic opioid addiction may be high (Boscarino et al., 2010; Manchikanti et al., 2010), and recent data indicate that clinically prescribed opioid exposure for as few as 5 days is associated with increased risk of long-term

opioid use (Shah et al., 2017). These findings suggest that opioids retain considerable abuse liability in pain patients, and this concern has triggered the implementation of more restrictive guidelines for opioid prescriptions (Dowell et al., 2016).

Despite evidence for iatrogenic addiction, there are still many within the opioid use field that interpret clinical results (often in the relatively acute setting) as evidence that pain may be protective against opioid addiction when analgesics are prescribed appropriately (American Academy of Pain Medicine 2004; The Pain Society 2004 Hunt and Urch, 2013). These notions are often reinforced by a concept known as pseudoaddiction, in which a patient may exhibit behaviors that are associated with addiction, including compulsive drug seeking and hoarding of medications, when opioid analgesics are prescribed inadequately (Heit, 2001; Greene and Chambers, 2015), but that disappear once pain is adequately controlled. The hypothesis that pain is protective against the abuse-related effects of opioids continues to face data that do not support or are in opposition of what it would predict, with rates of iatrogenic opioid addiction possibly higher in chronic pain patients (Ballantyne and LaForge, 2007; Anghelescu et al., 2013; Koyyalagunta et al., 2013; Barclay et al., 2014; Del Fabbro, 2014; Rauenzahn et al., 2017). It is not clear if pain alters opioid abuse liability or merely provides the occasion for opioid exposure.

The presence of a pain state may modulate the rewarding effects of opioid analgesics through several mechanisms. Both the analgesic and abuse-related effects of opioid drugs are mediated through the mu-opioid receptor (MOR), a G-protein coupled receptor that signals through Gi/o inhibition of adenylate cyclase (Metcalf et al., 1979). MORs are abundant in regions of the brain that are involved in reward (VTA, NAc, amygdala), analgesia (thalamus, PAG, RVM, and dorsal horn of the spinal cord), and motor coordination (primary somatosensory and motor cortex) (Stein et al., 2003). As noted earlier, pain can depress behavior and cause

correlated decreases in mesolimbic dopamine functioning. In a similar process, a pain state may inhibit the reward signaling in MOR-expressing neurons of the VTA, NAc, PAG, RVM, and amygdala when a MOR agonist is administered, reducing the abuse-related effect of an opioid drug (Figure I.3). Pain states also increase the production and release of β-endorphins, endogenous MOR agonists (Luan et al., 2017). With repeated activation by MOR agonists, desensitization and internalization of MORs can occur, leading to tolerance (Bourova et al., 2010; Groer et al., 2011). A chronic pain state may be sufficient to induce desensitization of MORs in brain regions associated with reward, blunting the abuse-related effect of MOR agonists when administered. Lastly, it has been argued that chronic pain states can have the opposite effect by enhancing the rewarding effects of analgesic drugs in creating conditions under which those drugs produce negative reward (associated with reversal of an aversive pain state) in addition to whatever positive rewarding effects they may also produce (Navratilova et al., 2015)

The presence of a pain state is not the only input that may be modulating the reward of opioid analgesics. Repeated MOR agonism from chronic administration of opioid analgesics may be the responsible for observations of tolerance to analgesic efficacy and to adverse effects such as nausea, emesis, and respiratory depression in addition to modulations in the rewarding effects of opioids. While tolerance to analgesia, nausea, and sedation has been well documented (Zakowski et al., 1992; Chu et al., 2012), tolerance does not appear to develop for the constipating and abuse-related effects of MOR agonists (Donner et al., 1998; Hojsted et al., 2013). One potential reason for this is related to the functioning of MORs themselves in different regions of the body responsible for different opioid effects. Density, neuron type, and signaling machinery may all play a role in the different degrees of tolerance to different opioid

effects observed with repeated opioid exposure (Connor et al., 2015). It can be hypothesized that repeated exposure to MOR agonists produces tolerance to sedative and/or negative affective (punishing) effects of the drug while keeping the positive affective (rewarding) effects intact, independent of the presence of a pain state. This hypothesis predicts the increased probability for abuse and dose escalation seen in patients treated with opioid analgesics (Hojsted et al., 2013; Shah et al., 2017).

Chemotherapy-induced neuropathic pain (CINP) is a common and dose-limiting side effect in the use of chemotherapeutic agents like paclitaxel for cancer treatment (Reeves et al., 2012; Speck et al., 2013; Seretny et al., 2014; Volkow and Koroshetz, 2017), and opioid agonists are commonly used to treat CINP (Plante and VanItallie, 2010), despite evidence for marginal therapeutic efficacy (Raja et al., 2002; McNicol et al., 2013; Argyriou et al., 2014). Iatrogenic opioid addiction is well documented in patients with CINP (Ballantyne and LaForge, 2007; Anghelescu et al., 2013; Koyyalagunta et al., 2013; Barclay et al., 2014; Del Fabbro, 2014; Rauenzahn et al., 2017), but as with pain in general, it is not clear if CINP in particular alters opioid abuse liability or merely provides an occasion for opioid exposure.

Preclinical studies investigating the effects of neuropathy on the rewarding effects of MOR agonists are limited by similar discrepancies to the their clinical counterparts discussed above in that a clear trend has not yet emerged (Table I.2). There is evidence that neuropathic manipulations may enhance, attenuate, or minimally change abuse-related endpoints compared to control subjects. Abuse liability testing in animals is conducted utilizing three assays with strong predictive validity of clinical drugs of abuse; conditioned place preference (CPP), operant self-administration (SA), and ICSS. In assays of CPP, rats exposed to neuropathic or sham stimuli were conditioned to associate the texture and visual cues of one chamber with a MOR agonist

and those of the other chamber with a control. Rats treated with cumulative doses of 4 mg/kg paclitaxel and 8 mg/kg oxaliplatin displayed no differences in place preference after conditioning with morphine (12 mg/kg), oxycodone (1.68 mg/kg), or fentanyl (0.051 mg/kg) compared to sham rats with increased preference observed in all rats (Mori et al., 2014). These results agree with later findings demonstrating no difference in preference scores between C57Bl6 mice treated with 32 mg/kg cumulative paclitaxel and mice treated with saline (Neelakantan et al., 2016). These findings differ from those obtained in rats exposed to intraplantar formalin, an inflammatory and neuropathic noxious stimulus, and in a surgical neuropathy model known as chronic constriction injury (CCI) of the common sciatic nerve. Formalin-treated rats expressed greater preference for the morphine-associated chamber after 20m/kg morphine exposure (Bardin et al., 2000). Exposure to lower doses of morphine (4, 8, and 16 mg/kg) produced a place preference in rats with injuries to the common sciatic nerve (CCI) but not in sham rats (Cahill et al., 2013). Then there are studies that have observed a decreased preference for MOR agonists in CPP assays. Mice and rats that underwent partial sciatic nerve ligation (pSNL) surgeries showed a lack of preference for morphine-associated chambers following 12 and 24mg/kg morphine exposure while rats that underwent sham surgeries displayed preference for morphine-associated chambers (Ozaki et al., 2002; Ozaki et al., 2003).

The evidence of neuropathic noxious stimuli impacting the reinforcing effects of morphine has also netted mixed results. In one study, drug-taking operant behavior was acquired at higher doses of MOR agonists in rats with ligated L5 and L6 spinal nerves (SNL) compared to sham animals. Heroin, methadone, morphine, fentanyl, and hydomorphone all displayed a potency shift to the right (less reinforcing) in nerve-ligated rats. However, this effect was abolished when animals were exposed to a descending order of self-administration (exposure to

higher doses first), suggesting that repeated exposure may eliminate any changes induced by the ligation surgery (Martin et al., 2007). However, in a different study where C57Bl6 mice were treated with 32mg/kg cumulative paclitaxel or saline, there were no differences observed in breakpoint for 0.1 (males) or 0.03 (females) mg/kg morphine infusions under a progressive ratio (Neelakantan et al., 2016)

The effects of a drug on ICSS have become a useful tool for prediction of that drug's potential abuse liability. ICSS procedures produce both high and low rates of responding that are dependent on the frequency of brain stimulation (Figure I.4). Many drugs of abuse increase or "facilitate" rates of ICSS reinforcement across lower frequencies of brain stimulation. Facilitation of ICSS is often interpreted as an abuse-related drug effect that can be tested in both the presence and absence of a pain state (Wise, 1996; Carlezon and Chartoff, 2007; Altarifi and Negus, 2011; Negus and Miller, 2014; Miller et al., 2015). ICSS procedures have a record of predictive validity similar to that of drug self-administration procedures for preclinical abuse-liability assessment, and most drugs that facilitate ICSS also have abuse liability in humans (Wise, 1996; Negus, 2013; Negus and Miller, 2014).

In an assay of ICSS in rats with ligated L5 and L6 spinal nerves and control rats, fentanyl, methadone, oxycodone, and morphine produced facilitation of ICSS in all groups. While the neuropathic manipulation did not prevent the abuse-related effect of MOR agonists in ICSS, it did shift the potency of fentanyl, methadone, oxycodone, and morphine to induce facilitation to the right (less reinforcing) roughly two-fold. Importantly, this surgical neuropathic manipulation did not change ICSS baseline behavior (Ewan and Martin, 2011b; Ewan and Martin, 2011a; Ewan and Martin, 2012)

The previous studies above involve acute administration of MOR agonists. One advantage of ICSS is that drug effects can be monitored during the earliest stages of drug exposure, and this is especially relevant with opioids because initial exposure produces little or no ICSS facilitation in drug-naïve subjects, but repeated daily exposure for as little as one week results in the gradual emergence of rewarding effects (Altarifi and Negus, 2011; Wiebelhaus et al., 2016). Little is known about the degree to which pain states might modify this trajectory of increasing opioid reward during initial opioid exposure; however, repeated exposure to an acute pain stimulus failed to modify this trajectory (Miller et al., 2015), and this agrees with the increased risk of long-term opioid abuse in patients that receive clinically prescribed opioid exposure for as few as 5 days (Shah et al., 2017).

While CPP and self-administration of MOR agonists involve activation of MORs throughout the central nervous system (both positive and negative effects), it is possible to explore neuropathy-induced changes in targeted regions such as VTA and NAc. In vivo microdialysis experiments in rats following paclitaxel treatment revealed no differences in morphine-induced increase of extracellular dopamine within NAc (Mori et al., 2014), a critical component of the rewarding effects of morphine. However, in rats following a surgical neuropathic manipulation (pSNL), in vivo microdialysis found a suppression of morphine-induced extracellular dopamine release in NAc compared to sham rats (Ozaki et al., 2002). The same study also investigated the effects of a surgical neuropathy on [^{35}S]GTPγS binding stimulated by morphine on VTA membranes. Agonist-stimulated [^{35}S]GTPγS binding is an assay that tests functional activity at a particular receptor. Neuropathic manipulation attenuated MOR agonist-stimulated [^{35}S]GTPγS binding on VTA membranes compared to sham rats (Ozaki et al., 2002)

Table I.2: Preclinical assessment of opioid reward following neuropathic manipulation

Sex and Species	Insult	Acute Opioid (Total Exposure)	Test	Result	Insult effect on abuse liability	Reference
Male Sprague-Dawley rats	Paclitaxel (4mg/kg) Oxaliplatin (8mg/kg)	Morphine (12mg/kg) Oxycodone (1.68mg/kg) Fentanyl (0.05mg/kg)	CPP	All drugs produced preference in control and chemotherapy rats	⟷	(Mori et al., 2014)
Male Sprague-Dawley rats	Oxaliplatin (8mg/kg)	Morphine (12mg/kg) Oxycodone (1.68mg/kg) Fentanyl (0.05mg/kg)	CPP	All drugs produced preference in control and chemotherapy rats	⟷	(Mori et al., 2014)
Male Long-Evans rats	Formalin (50ul, 2.5%)	Morphine (20mg/kg)	CPP	Increased morphine preference in formalin	↑	(Bardin et al., 2000)
Male Long-Evans rats	CCI	Morphine (4, 8, 16mg/kg)	CPP	Increased morphine preference in CCI	↑	(Cahill et al., 2013)
Male ICR mice	pSNL	Morphine (12, 24 mg/kg)	CPP	Decreased morphine preference in pSNL rats	↓	(Ozaki et al., 2002)
Sprague-Dawley rats	pSNL	Morphine (9, 30, 90nmol i.c.v.)	CPP	Decreased morphine preference in pSNL rats	↓	(Ozaki et al., 2003)
C57Bl6 Mice	Paclitaxel (32mg/kg)	Morphine (0.9, 7.5, and 30.0 mg/kg)	CPP SA	No increased preference or in SA breakpoint	⟷	(Neelakantan et al., 2016)

Sex and Species	Insult	Acute Opioid (Total Exposure)	Test	Result	Insult effect on abuse liability	Reference
Male Fisher rats	SNL	Heroin, methadone, morphine, fentanyl, hydro-morphone	SA	MOR agonists displayed a potency shift to the right in nerve-ligated rats		(Martin et al., 2007)
Male Fisher rats	SNL	Fentanyl (0.06 mg/kg), methadone (6mg/kg), oxycodone (3mg/kg), and morphine (6mg/kg)	ICSS	Shift in potency of MOR agonists to induce facilitation to the right roughly two-fold		(Ewan and Martin, 2011a; Ewan and Martin, 2012; Ewan and Martin, 2014)

5. Sex Differences in Pain and Opioid Abuse

Paclitaxel, vincristine, oxaliplatin, and bortezomib are used to treat a variety of different cancers, some of which predominately affect males (nasopharyngeal) and others that predominately affect females (breast and ovarian carcinoma), and these chemotherapies produce a pain associated-neuropathy and depression of behaviors in both sexes. The degree to which there are sex differences in undesirable effects of chemotherapy is relatively unknown; however, both epidemiological and human laboratory studies have reported lower pain thresholds, less pain tolerance, and greater evoked pain in women compared with men (Fillingim et al., 2009; Ruau et al., 2012; Bartley and Fillingim, 2013). Few studies have investigated the potential for sex differences in the effects of chemotherapy-induced neuropathy, CINP, or depression of behavior in a preclinical setting, only reporting a difference among sexes on cold allodynia at

one dose of paclitaxel (Hwang et al., 2012, Naji-Esfahani et al., 2016) and increased vincristine-induced olfactory epithelium lesions in females (Kai et al., 2006), while others have noted sex differences in pain and analgesia in other preclinical models of pain (Craft, 2003; Mogil, 2012). Although not well understood and absent in many examples of clinical pain and preclinical antinociception, sex differences in pain are often attributed to gonadal steroid hormones (Craft et al., 2004). One of the possible mechanisms for the effects of gonadal hormones on pain perception is neuromodulation in response to repeated exposure to noxious stimuli, through changes in opioid receptor functioning. For example, in an assay of formalin-induced paw licking, female rats had higher frequencies of paw licking than males, but central administration of estradiol, a primarily female gonadal hormone, in males abolished this sex difference (Aloisi and Ceccarelli, 2000). Interestingly, the effect of estradiol in male rats could be blocked by naloxone, suggesting a potential modification of opioid receptors. Additional evidence of the modulation of opioid receptors by estradiol include mu-opioid receptor (MOR) internalization in regions of the brain such as medial preoptic nucleus and posterodorsal medial amygdaloid nucleus (Eckersell et al., 1998) while producing increased MOR binding sites in hypothalamus and thalamus (Dondi et al., 1992).

In addition to the presumed pro-nociceptive effects of estrogen hormones modulating the perception of pain in males and females, sex differences have also been observed with the acute effects of MOR agonist drugs in preclinical studies (Cicero et al., 2003; Lynch, 2006; Craft, 2008; Lynch et al., 2013). Of note, females often appear to be more sensitive than males to the abuse-related effects of MOR agonists (Craft, 2008). In self-administration studies with rodents, females acquired behavior for heroin and morphine faster than males (Cicero et al., 2003; Lynch, 2006; Lynch et al., 2013), and in a conditioned place preference study, females expressed greater

place preference for high doses of morphine (Cicero et al., 2000). However, it is unclear as to whether the enhanced abuse-related effects of MOR agonists in females are due to modulation of MORs in regions of the brain associated with reward or with a sex-related resistance to opioid-induced sedation. Previous observations of increased sensitivity of males to the sedative effects of opioids in assays of locomotion in males compared to females (Holtman et al., 2004; Craft et al., 2006) suggest that the sex differences detected with MOR agonists could be a result of increased sedation in males that mask similar abuse-related effects between the sexes. Although these observations of enhanced abuse-related effects in females with acute MOR agonist administration continue to be explored, the potential for sex differences in abuse liability with repeated or chronic MOR agonist administration is not known. Regardless of mechanism or brain regions affected, these preclinical findings map on well with the clinical observations in opioid abuse-related endpoints. Women have an earlier age of initiation of opioid substance abuse and a more rapid progression from initial use to dependence (Anglin et al., 1987; Hernandez-Avila et al., 2004) despite no large differences in overall prevalence of opioid use disorder (Becker et al., 2008; Manubay et al., 2015; Graziani and Nistico, 2016; Serdarevic et al., 2017).

6. Overview of Dissertation Studies

The overall goal of this dissertation was to evaluate the effect of chemotherapy treatment on motivated behaviors and opioid reward. This was achieved by conducting experiments designed to answer the following questions. (1) Do chemotherapy treatments that produce pain-stimulated hypersensitive withdrawal responses also produce pain-related decreases in motivated behavior? (2) If so, can these effects be reversed by morphine, a clinically used analgesic? (3) Are there differences in the severity and time course of effects with administration of the four

neuropathic classes of chemotherapeutics? (4) Does chemotherapy protect against the abuse-related effects of morphine? The approach of the studies and data presented in this dissertation were based on the following hypothesis: Administration of chemotherapy induces a pain state (CINP) that decreases mesolimbic dopamine signaling, resulting in depression of motivated behaviors, and morphine administration can restore chemotherapy-induced behavioral depression while producing abuse-related effects with repeated morphine exposure.

Chapter II of this dissertation describes results from my first publication (Legakis et al., 2018). Chapter II compares effects of paclitaxel treatment in rats on (a) mechanical sensitivity of paw-withdrawal responses, and (b) positively reinforced operant responding maintained by electrical brain stimulation in an ICSS assay or by food pellets in an assay of food-maintained responding. There were three main findings. First, paclitaxel doses sufficient to produce mechanical hypersensitivity did not reliably depress ICSS in male or female rats. Second, analysis of data from individual rats indicated that the degree of behavioral suppression in ICSS did not correlate with mechanical sensitivity. The lack of correlation between mechanical sensitivity and behavioral suppression suggests that mechanical hypersensitivity does not cause behavioral suppression, may have different underlying mechanisms than behavioral suppression, and may not serve as a useful surrogate measure for clinically relevant signs of behavioral depression in CINP. Third, food-maintained responding was more sensitive to detect chemotherapy-induced depression of motivated behavior than ICSS. Accordingly, the studies in Chapter III utilized food-maintained responding to compare the severity and time course of effects by paclitaxel, vincristine, oxaliplatin, and bortezomib on mechanical hypersensitivity and motivated behavior.

Chapter III of this dissertation describes results that are currently in preparation for submission. Chapter III compares the effects of the four neuropathic classes of chemotherapeutics using paclitaxel, vincristine, oxaliplatin, and bortezomib on mechanical hypersensitivity and motivated behavior. There were three main findings. First, differential effects were observed with the four different chemotherapeutics. Paclitaxel produced severe and sustained decreases in mechanical sensitivity thresholds and weak but significant decreases at later time points in food-maintained responding. Vincristine produced weak but sustained decreases in mechanical sensitivity thresholds and severe but transient decreases in food-maintained responding. Oxaliplatin produced severe and sustained decreases in mechanical sensitivity thresholds but weak and transient decreases in food-maintained responding. Bortezomib produced weak but sustained decreases in mechanical sensitivity thresholds and weak and transient decreases in food-maintained responding. Second, depression of food-maintained behavior was not observed for any chemotherapy, including paclitaxel, one month after initiation of treatment when demand for food was further evaluated using a behavioral economics approach in which the "cost" of food was progressively increased. Only paclitaxel administration was sufficient to produce both mechanical allodynia and long-term decreases in food-maintained responding, and even for paclitaxel, effects on food-maintained responding were small and observed with only one "cost" (i.e. with only one fixed-ratio value) of food reward. Third, morphine was effective to reverse paclitaxel-, vincristine-, oxaliplatin-, and bortezomib-induced mechanical hypersensitivity but was unable to reverse paclitaxel-induced behavioral depression of food-maintained responding. This result further distinguishes the possible mechanisms underlying hypersensitivity and behavioral depression and may illustrate

the limited efficacy of MOR agonists to treat CINP. Due to the results in Chapter III, paclitaxel was selected for the evaluation of chemotherapy effects on opioid reward in Chapter IV.

Chapter IV of this dissertation describes results from my second publication (Legakis and Negus, 2018). Chapter IV compared the effects of repeated morphine treatment on ICSS and mechanical hypersensitivity in male and female rats treated with paclitaxel. There were three main findings. First, as in Chapter II, paclitaxel produced sustained mechanical hypersensitivity but no significant change in baseline ICSS performance in both males and females. Second, initial morphine treatment dose-dependently alleviated paclitaxel-induced mechanical hypersensitivity in both sexes, and repeated morphine produced modest but significant tolerance to this antinociceptive effect. Third, initial morphine treatment produced greater abuse-related ICSS facilitation in females than males, and repeated morphine treatment enhanced ICSS facilitation in both male and female rats treated with either paclitaxel or its vehicle. Overall, these results suggest that this model of paclitaxel-induced neuropathy does not alter the trajectory of increasing opioid reward that occurs during initial exposure to repeated morphine. More generally, these results suggest that CINP is not protective against opioid reward, and repeated morphine treatment may simultaneously produce both tolerance to analgesic effects and increased vulnerability to iatrogenic opioid addiction.

The major conclusion of my work is that chemotherapy-induced neuropathy can produce severe mechanical hypersensitivity and weak behavioral depression in rats, and these two effects did not correlate with each other. Morphine is sufficient to reverse mechanical hypersensitivity but not behavioral depression. These findings suggest that the mechanisms producing hypersensitivity and behavioral depression may be different. The class of chemical neuropathic agent in preclinical research is a determinant of its behavioral effects as severity and time course

were different with paclitaxel, vincristine, oxaliplatin, and bortezomib. Most importantly, chemotherapy treatment did not protect against the rewarding effects of morphine, which increased with increasing exposure. This dissertation not only extends the literature on chemotherapy and its interactions with opioid abuse but also provides novel assessment of chemotherapy on motivated behaviors, evaluates four classes of chemotherapeutic agents on commonly and uncommonly tested endpoints, and provides evidence to proceed with caution when prescribing opioids for CINP due to the risk for abuse and potentially underwhelming clinical efficacy to restore function or behavioral depression.

Chapter II: Lack of Paclitaxel Effects on Intracranial Self-Stimulation in Male and Female Rats: Comparison to Mechanical Sensitivity

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Introduction

The goal of the present study was to test the hypothesis that paclitaxel treatment regimens sufficient to produce mechanical hypersensitivity in rats would also produce depression of operant responding maintained by either (a) electrical brain stimulation in an assay of intracranial self-stimulation (ICSS), or (b) food delivery in an assay of food-maintained responding. The density of intra-epidermal nerve fibers in hindpaw was also evaluated as previous studies have shown that decreases in fiber density correlate with mechanical hypersensitivity (Boyette-Davis et al., 2011a), but the relationship of fiber density to behavioral depression has not been examined. Studies were conducted in both males and females because paclitaxel is used to treat cancer in both sexes, sex differences have been reported for some pain states in patients (Ruau et al., 2012; Bartley and Fillingim, 2013), and preclinical studies have reported sex differences in some paclitaxel effects (Hwang et al., 2012).

Methods

Subjects

Studies were conducted in adult male (51) and female (12) Sprague Dawley rats with initial weights ranging from 360 to 468 g in males and 236 to 298g in females. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM in an AAALAC International-accredited housing facility. Males in studies of food-maintained responding had access to 45 mg food pellets (BioServ Dustless Precision Pellets, Product#

F0042, Flemington, NJ) during operant behavior sessions, and they were given access to unlimited water and 7g per day of standard chow diet (Teklad standard diet - 19% protein, Envigo, Madison, WI) after experimental sessions. For all other rats, standard chow diet and water were available ad libitum in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

Drugs

Paclitaxel was obtained as a clinically available 6.0 mg/ml solution (TEVA Pharmaceuticals, North Wales, PA) and diluted in vehicle (8.3% ethanol, 8.3% Cremophor EL, and 83.4% saline) to final concentrations of 0.335, 1.0, and 3.0 mg/ml. All rats were injected intraperitoneally (i.p.) on four alternate days (Days 1, 3, 5, and 7) with vehicle or a given dose of paclitaxel (0.67, 2.0, or 6.0 mg/kg) using an injection volume of 2 ml/kg. These dosing regimens resulted in cumulative doses of 2.68, 8.0, and 24.0 mg/kg of paclitaxel.

Intracranial self-stimulation (ICSS)

Surgery. Thirty-nine male and twelve female rats were anesthetized with inhaled isoflurane (2.5-3% in oxygen; Webster Veterinary, Phoenix, AZ) and implanted with electrodes (Plastics One, Roanoke, VA) in the left medial forebrain bundle at the level of the lateral hypothalamus using previously published procedures and coordinates (Males: 2.8 mm posterior to bregma, 1.7 mm lateral to midsagittal suture, 8.8 mm below skull surface; Females: 3.8 mm posterior to bregma, 1.6 mm lateral to midsagittal suture, and 8.7 mm below skull surface) (Lazenka et al., 2016a; Lazenka et al., 2016b). The electrode was secured to the skull with orthodontic resin and skull screws. Ketoprofen (5 mg/kg i.p.; Spectrum Chemical, New

Brunswick, NJ) was administered immediately and 24 hours after surgery as a postoperative analgesic, and rats recovered for 7 days prior to initiation of ICSS training.

Apparatus. Studies were conducted in sound-attenuating boxes containing modular acrylic and metal test chambers (29.2 x 30.5 x 24.1 cm; Med Associates, St Albans, VT). Each chamber contained a response lever, three stimulus lights (red, yellow, and green) centered above the lever, a 2-W house light, and an ICSS stimulator. Electrodes were connected to the stimulator via bipolar cables routed through a swivel commutator (Model SL2C, Plastics One, Roanoke, VA). Control of stimulus delivery in the operant chamber and collection of data on lever presses were accomplished with a computer, interface, and custom software (Med PC-IV, Med Associates).

Training. Rats were trained to respond for electrical brain stimulation using procedures identical to those described previously (Leitl et al., 2014a). Briefly, a white house light was illuminated during behavioral sessions, and responding under a fixed-ratio (FR) 1 schedule produced a 500-msec train of 0.1-msec square-wave cathodal pulses together with 500-msec illumination of stimulus lights over the lever. Responding during brain stimulation had no scheduled consequences. The terminal schedule consisted of sequential 10-min components. Each component consisted of 10 1-min trials, and the available brain-stimulation frequency decreased in 0.05 log Hz increments from one trial to the next (158-56 Hz). Each frequency trial consisted of a 10-sec timeout, during which five noncontingent stimulations were delivered at the frequency available during that trial, followed by a 50-sec "response" period, during which responding resulted in electrical stimulation. Training continued with presentation of three sequential components per day until the following two criteria for stable responding were met for

three consecutive days: (1) ≤5% variability in the maximum rate of reinforcement in any trial, and (2) ≤10% variability in the total number of stimulations per component.

Testing. Once responding stabilized, a 29-day testing protocol began (Figure II.1a). Three-component ICSS operant behavioral sessions were conducted daily (with occasional exceptions on weekends) throughout the 29-day test period, and vehicle or paclitaxel was administered 2 hr before behavioral sessions on Days 1, 3, 5, and 7. Studies were conducted in three phases. First, four groups of male rats (N=6-7 per group) were used to evaluate effects of vehicle and three different paclitaxel doses (0.67, 2.0, or 6.0 mg/kg/day). The initial paclitaxel dose-effect study revealed a small but non-significant decrease in ICSS after treatment with 2.0 mg/kg/day paclitaxel and severe weight loss after 6.0 mg/kg/day paclitaxel. Mean data and standard deviations for effects of vehicle and 2.0 mg/kg/day paclitaxel on ICSS on Day 29 in the dose-effect study were used for power analysis sample-size estimates required to detect significance for the paclitaxel effect size given an alpha of 0.05, power of 0.8, allocation ratio of 1.5 (i.e. 50% more rats in the paclitaxel treatment group given variability in paclitaxel effects), and use of a one-tailed t-test (given the prediction that paclitaxel would reduce ICSS) (Faul et al., 2007). The computed sample sizes were 12 vehicle-treated and 18 paclitaxel-treated rats. Accordingly, a follow-up study was conducted to add six vehicle- and 12 paclitaxel-treated rats to the initial samples. Lastly, to assess potential sex differences in paclitaxel effects, two groups of female rats (N=6 per group) were treated with vehicle or 2.0 mg/kg/day paclitaxel. All rats were weighed before each operant behavioral session. In addition, mechanical sensitivity was assessed before and on Days 8, 15, 22, and 29 after initiation of paclitaxel treatment (methods described below). On Day 29, rear paws were collected from a subset of male and female rats for assessment of intra-epidermal nerve fiber density (methods described below).

Data analysis. The first baseline component of each test session was considered to be a "warm up" component, and data were discarded. Data from the remaining two components were analyzed as previously described (Leitl et al., 2014b; Negus and Miller, 2014). The primary dependent measure was the total number of reinforcements per component (i.e. the total number of stimulations delivered across all brain-stimulation frequencies during each component). Data for the final three training days prior to vehicle/paclitaxel treatment were averaged to provide a mean pre-paclitaxel baseline measure of reinforcements per component in each rat. Once paclitaxel treatment was initiated, the number of reinforcements per component was determined daily in each rat and expressed as a percentage of that rat's pre-paclitaxel baseline using the equation: % Baseline Reinforcements per Component = (Number Reinforcements per Component on a Test Day ÷ Pre-Paclitaxel Baseline Reinforcements per Component) x 100. Changes in ICSS performance over time were then averaged across rats and evaluated in three ways. In the first approach, data from each day of the study were analyzed by repeated-measures two-way ANOVA, with paclitaxel dose and treatment day as the two factors. A significant ANOVA was followed by the Holm-Sidak post-hoc test. In the second approach, the same data were analyzed using linear regression across days within each treatment group. Model comparison was used to determine whether treatment impacted regression coefficients (β_1) by an extra sum-of-squares F-test (Motulsky and Christopoulous, 2003; Hutsell et al., 2016). In the final approach, data from the last day of the study (Day 29) were compared across paclitaxel doses using one-way ANOVA. For all approaches, and for all other analyses described below, statistical analysis was conducted using Prism 7.0 (Graphpad Software Inc., San Diego, CA), and the criterion for statistical significance was $p<0.05$.

A secondary and more granular measure of ICSS performance was the reinforcement rate

in stimulations per frequency trial. Raw reinforcement rates for each rat from each trial were converted to percent maximum control rate (%MCR), with MCR defined as the mean of the maximal rates observed at any trial during the three pre-paclitaxel baseline sessions. Thus, %MCR values for each trial were calculated as {(reinforcement rate during a frequency trial ÷ MCR) × 100}. %MCR values were then averaged across rats and analyzed by repeated-measures two-way ANOVA, with ICSS frequency and treatment day as the two factors. A significant ANOVA was followed by the Holm-Sidak post-hoc test.

Food-maintained operant responding

Apparatus. Operant conditioning chambers similar to those described above (Med Associates) were housed in sound-attenuating boxes and equipped with a response lever, three stimulus lights centered above the lever, a 2-W house light, and a pellet dispenser that delivered 45 mg food pellets (BioServ Dustless Precision Pellets, Product# F0042, Flemington, NJ) to an aperture beside the lever. As with ICSS, control of stimulus delivery in the operant chamber and collection of data on lever presses were accomplished with a computer, interface, and custom software (Med PC-IV, Med Associates).

Training. Onset of the house light signaled the beginning of 30-min behavioral sessions during which lever presses produced delivery of a food pellet under a FR schedule. The FR was gradually increased from FR 1 to FR 5, and after each pellet delivery, there was a 0.5-sec time out period during which the lever lights were illuminated and responding had no scheduled consequences. Training continued until the following criteria for stable responding were met for three consecutive days: (1) subjects earned ≥75 reinforcements/session, and (2) the number of reinforcements/session on each day varied by ≤5% of the running mean.

Testing. Once responding stabilized, a 29-day testing protocol began similar to that used for ICSS studies. Behavioral sessions were conducted daily (with occasional exceptions on weekends) throughout the 29-day test period, and vehicle or 2.0 mg/kg paclitaxel was administered 2 hr before behavioral sessions on Days 1, 3, 5, and 7. Rats were weighed before each operant behavioral session, and chow allotments were provided two hours following behavioral sessions. In addition, mechanical sensitivity was assessed before and on Days 8, 15, 22, and 29 after initiation of paclitaxel treatment (methods described below).

Data analysis. The primary dependent measure for studies of food-maintained operant responding was the total number of reinforcements/session. Data from the final three training days prior to initiation of vehicle/paclitaxel treatment were averaged to produce a mean pre-paclitaxel baseline measure of reinforcements/session for each rat. Once paclitaxel treatment was initiated, the number of reinforcements/session was determined daily in each rat and expressed as a percentage of that rat's pre-paclitaxel baseline using the equation: % Baseline Reinforcements = (Number Reinforcements on a Test Day ÷ Pre-Paclitaxel Baseline Reinforcements) x 100. Changes in food-maintained responding over time were then averaged across rats and evaluated as in ICSS studies described above. First, data were analyzed by repeated-measures two-way ANOVA, with treatment and day as the two factors. Second, reinforcements/session were analyzed using linear regression across days within each treatment group. Lastly, data from the last day of treatment (Day 29) were compared by t-test.

Mechanical sensitivity testing with von Frey filaments

On days when mechanical sensitivity was assessed, testing was conducted approximately 1 hr after conclusion of the operant behavioral session on that day. Rats were first placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to

acclimate for at least 20 mins before exposure to the mechanical stimuli. Subsequently, von Frey filaments (ranging from 0.4 to 15.0g and increasing in ~0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar surface of each paw, and the threshold stimulus to elicit paw withdrawal was determined in log grams using the "up-down" method as previously described (Chaplan et al., 1994; Leitl et al., 2014b). On each test day, data were averaged across paws within each rat and then across rats. Changes in threshold over time were analyzed by two-way ANOVA, with paclitaxel dose and treatment day as the two factors, and a significant ANOVA was followed by the Holm-Sidak post-hoc test.

Immunohistochemistry

Hind paws were analyzed for intra-epidermal nerve fiber (IENF) density from a subset of 8 males (N=3 vehicle, N=3 2.0 mg/kg/day paclitaxel, N=2 6.0 mg/kg/day paclitaxel) and 6 females (N=3 vehicle, N=3 2.0 mg/kg/day paclitaxel) from ICSS studies. Vehicle-treated rats were selected randomly. Paclitaxel-treated rats were selected to represent the range of effects on mechanical sensitivity and ICSS. On Day 29 after completion of all behavioral studies, rats were anesthetized by intraperitoneal injection of 3 g/kg of Euthasol (Patterson Veterinary, Greeley, CO) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Footpad skin was collected, fixed for an additional 24 hours in 4% paraformaldehyde, and stored in 0.1 M phosphate-buffered saline (PBS). Footpad skin was embedded in paraffin and sectioned at 25 μm increments perpendicular to the epidermal surface.

To identify intra-epidermal nerve fibers, samples were immunostained using an immunoperoxidase method (males) or an immunofluorescence method (females). Immunoflourscence was used for females, which were studied later, in an attempt to improve contrast of intra-epidermal nerve fibers within epidermal tissue; however there were no apparent

differences in the immunoreactivity, quantification, or results, so results from male and female samples were combined. For male samples, deparaffinized sections were washed in PBS, and endogenous peroxidase activity was quenched with 1% hydrogen peroxide in methanol for 20 min. Following a PBS wash, sections were incubated for 1 hr in blocking solution consisting of 5% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton-X-100 in PBS and incubated overnight at 4°C in primary antibody for PGP 9.5 (Fitzgerald Industries International, cat# 70R-30722, North Acton, MA) at a concentration of 1:1000 in blocking solution. Samples from females were treated similarly except for omitting the quenching step. For male samples, sections were washed with PBS for 30 min and then incubated with peroxidase-conjugated secondary antibody (Vector Laboratories) diluted to 1:250 in blocking solution for 1 hr at room temperature. Antibody binding was visualized using the Vectastain kit (Vector Laboratories) and incubation with diaminobenzadine (Sigma-Aldrich, St. Louis, MO). For female samples, sections were washed with PBS for 30 min and then incubated for 1 hr with secondary antibody conjugated with Alexa Flour 594 ® at a dilution of 1:250 (Life Technologies – cat# A11037, Eugene, OR).

For each animal, the number of intra-epidermal nerve fibers in approximately a one-centimeter length of epidermis from four sections was counted using light microscopy (Olympus CH-2, Center Valley, PA) for males or fluorescence microscopy (Zeiss Axio Imager A1, Carl Zeiss, AG, Germany) for females. The investigator was blind to the treatment. Data were expressed as fibers/mm, averaged across sections for a given rat, and then averaged across rats within a given treatment. Treatment effects were compared using a one-way ANOVA and a Dunnett's post hoc test.

Correlational analysis across endpoints

For rats in ICSS studies, correlations were evaluated for individual data from the last day of treatment (Day 29) for mechanical sensitivity, ICSS performance, and intra-epidermal nerve fiber density. For rats in studies of food-maintained responding, correlations were evaluated for individual data from the last day of treatment (Day 29) for mechanical sensitivity and rates of food-maintained responding.

Results

Paclitaxel effects on body weight, mechanical sensitivity, and ICSS in male rats

For male rats used in ICSS studies, the baseline body weight was 411.2 ± 13.3 g, the baseline mechanical sensitivity threshold was 1.14 ± 0.02 log g, and baseline measures of ICSS performance were 153.5 ± 16.4 stimulations per component with maximum control rates (MCR) of 56.3 ± 4.0 stimulations per trial. Figure II.1 b-d shows the time course of changes in body weight, mechanical sensitivity, and ICSS performance during and after repeated treatment with vehicle or different doses of paclitaxel (0.67, 2.0, or 6.0 mg/kg/day). Body weight increased over time in the vehicle-treated group, and similar weight gain was observed in rats treated with 0.67 and 2.0 mg/kg/day paclitaxel. Seven rats were treated with 6.0 mg/kg/day paclitaxel, but four of these rats lost ≥ 20% of their baseline body weight during the initial week of paclitaxel treatment and were euthanized in accordance with moribundity criteria in the animal use protocol. Data from these rats were excluded from all subsequent analyses, and their data are not included in Figure II.1. The remaining three rats also lost weight, and body weight in these rats was significantly lower than in vehicle-treated rats for Days 7-14 and Day 16; however, the

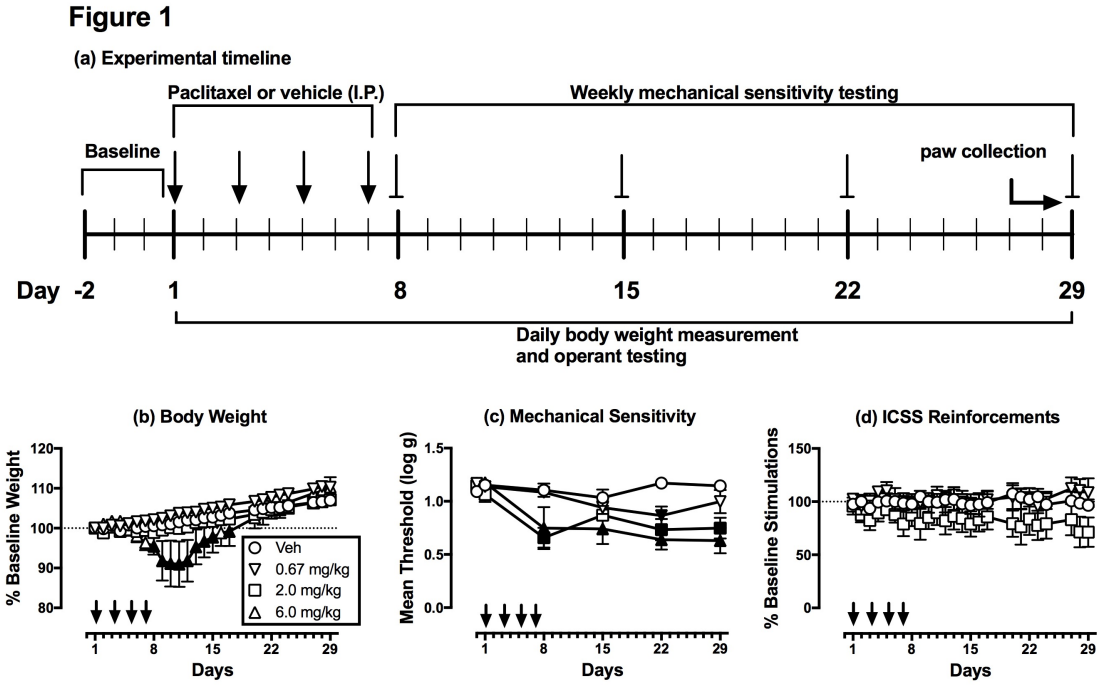

Figure II.1: Dose-dependent paclitaxel effects in male rats. Panel a shows the experimental timeline for treatment administration and data collection. Panels b-d show effects of different paclitaxel doses on different experimental endpoints. Horizontal axes: Time in days relative to initiation of vehicle/paclitaxel treatment on Day 1. Arrows indicate vehicle/paclitaxel treatment days. Vertical axes: (b) % baseline body weight, (c) mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g, and (d) ICSS performance expressed as % baseline number of brain-stimulation reinforcements earned per 10-min component. All points show mean±SEM for six rats (vehicle, 0.67 and 2.0 mg/kg/day paclitaxel) or three rats (6.0 mg/kg/day paclitaxel). Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (b) Significant main effects of treatment [$F(3,17)=3.33$; $p=0.044$] and time [$F(24,408)=83.64$; $p<0.0001$], and a significant interaction [$F(72,408)=6.73$;

$p<0.0001$]. (c) Significant main effects of treatment [$F(3,17)=15.54$; $p<0.0001$] and time [$F(5,85)=13.68$; $p<0.0001$], and a significant interaction [$F(15,85)=3.16$; $p=0.0004$]. (d) No significant main effects of treatment [$F(3,17)=1.14$; $p=0.362$] or time [$F(24,408)=0.74$; $p=0.815$], and no significant interaction [$F(72,408)=0.87$; $p=0.768$].

magnitude of weight loss in these rats did not reach the 20% criterion for euthanasia, and their weights recovered to control levels by the end of the 29-day study.

Paclitaxel also produced dose- and time-dependent decreases in mechanical sensitivity thresholds, and paclitaxel was both more potent and longer acting to produce mechanical hypersensitivity than weight loss. Thus, mechanical hypersensitivity was significant on Day 22 in rats treated with 0.67 mg/kg/day paclitaxel, Days 8, 22 and 29 in rats treated with 2.0 mg/kg/day paclitaxel, and all days of testing (Days 8, 15, 22 and 29) in rats treated with 6.0 mg/kg/day paclitaxel.

Despite producing significant dose-dependent weight loss and mechanical hypersensitivity, no dose of paclitaxel was sufficient to significantly decrease ICSS responding as determined by two-way ANOVA. However, two other findings provided evidence for at least some paclitaxel-induced ICSS depression. First, inspection of individual data indicated that ICSS was depressed in some rats (see below for details). Second, linear regression analysis of ICSS rates over time indicated a significant difference between the slopes (95%CLs) of the ICSS time-course data after repeated 2.0 mg/kg paclitaxel [negative slope = -0.51 (-0.71 to -0.30)] compared to repeated vehicle [slope no different from zero = 0.07 (-0.08 to 0.23)]. However, the ICSS time-course data after 0.67 mg/kg/day paclitaxel [slope = 0.18 (-0.05 to 0.42)] and 6.0 mg/kg paclitaxel [slope = 0.27 (-0.20 to 0.75) were not different from vehicle or from a value of zero.

To further explore paclitaxel effects on ICSS in male rats, a second study was conducted to increase the number of subjects to N=12 for vehicle treatment and to N=18 for 2.0 mg/kg/day paclitaxel. Figure II.2a-c shows results from all male rats treated with vehicle and 2.0 mg/kg/day paclitaxel. In this larger sample, 2.0 mg/kg/day paclitaxel produced significant but modest

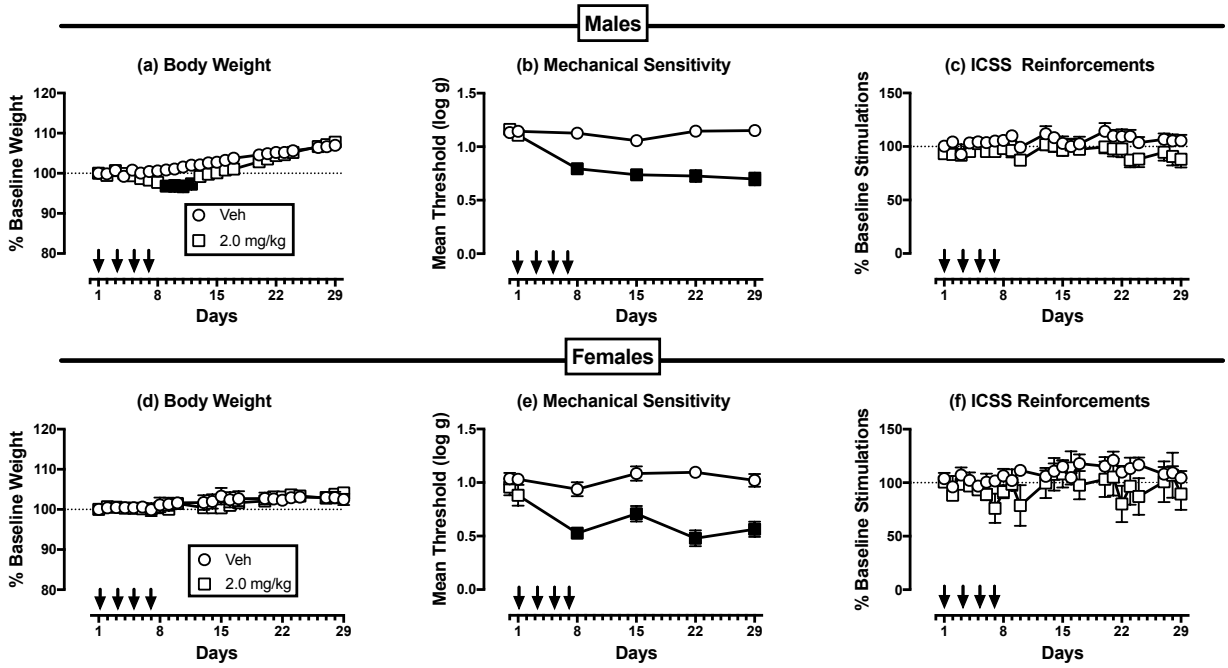

Figure II.2: Effects of vehicle and 2.0 mg/kg/day paclitaxel on all male and female rats included in ICSS studies. Panels a-c show vehicle (N=12) and 2.0mg/kg/day paclitaxel (N=18) effects in males. Panels d-f show vehicle (N=6) and 2.0mg/kg/day paclitaxel (N=6) effects in females. Horizontal axes: Time in days relative to initiation of vehicle/paclitaxel treatment on Day 1. Arrows indicate vehicle/paclitaxel treatment days. Vertical axes: (a, d) % baseline body weight, (b, e) mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g, and (c, f) ICSS performance expressed the % baseline number of brain-stimulation reinforcements earned per 10-min component. All points show mean±SEM, and filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) No significant main effect of treatment [$F(1,28)=3.58;p=0.069$], but a significant effect of time [$F(24,672)=47.68$; $p<0.0001$], and a significant interaction [$F(24,672)=4.33$; $p<0.0001$]. (b) Significant main effects of treatment [$F(1,28)=51.52$; $p<0.0001$] and time [$F(5,140)=17.83$;

$p<0.0001$], and a significant interaction [$F(5,140)=15.32$; $p<0.0001$]. (c) No significant main effect of treatment [$F(1,28)=2.36$;$p=0.136$], a significant effect of time [$F(22,616)=1.76$; $p=0.018$], and no significant interaction [$F(22,616)=0.90$; $p=0.601$]. (d) No significant main effect of treatment [$F(1,10)=0.06$; $p=0.818$], a significant effect of time [$F(22,220)=6.08$; $p<0.0001$], and no significant interaction [$F(22,220)=1.044$; $p=0.412$]. (e) Significant main effects of treatment [$F(1,10)=35.63$; $p<0.0001$] and time [$F(5,50)=7.11$; $p<0.0001$], and a significant interaction [$F(5,50)=6.37$; $p<0.0001$]. (f) No significant main effects of treatment [$F(1,10)=1.37$; $p=0.269$] or time [$F(22,220)=1.28$; $p=0.190$], and no significant interaction [$F(22,220)=0.78$; $p=0.751$].

weight loss from Days 9-12 (Figure II.2a), significant and sustained mechanical hypersensitivity throughout testing (Figure II.2b), and decreased ICSS in some rats (see below); however, paclitaxel still failed to significantly alter mean ICSS performance as assessed by two-way ANOVA (Figure II.2c). Moreover, in contrast to the first study, linear regression indicated no effect of paclitaxel treatment on ICSS. Specifically, the slope (95%CLs) of the time-course data for ICSS after 2.0 mg/kg/day paclitaxel [-0.12 (-0.35 to 0.11)] did not differ from zero or from the slope of the vehicle data [0.22 (0.00 to 0.44)]. Overall, these studies indicated that paclitaxel in male rats was most potent and effective to produce mechanical hypersensitivity, less potent to reduce body weight, and least potent and effective to reduce ICSS performance.

As one final indication of the weak effects of paclitaxel treatment on ICSS performance in male rats, Figure II.3a-b compares full ICSS frequency-rate curves at baseline and on Day 29 in all males treated with vehicle or 2.0 mg/kg/day paclitaxel. Two-way ANOVA did not indicate a significant main effect of treatment or an interaction between frequency and treatment for either vehicle or paclitaxel.

Paclitaxel effects on body weight, mechanical sensitivity, and ICSS in female rats

For all female rats used in ICSS studies, the baseline body weight was 263.8 ± 12.7 g, the baseline mechanical sensitivity threshold was 1.00 ± 0.10 log g, and baseline measures of ICSS performance were 124.5 ± 24.5 stimulations per component with maximum control rates (MCR) of 53.4 ± 6.5 stimulations per trial. T-test analysis indicated that at baseline, females had significantly lower body weights ($p<0.0001$), mechanical sensitivity thresholds ($p=0.001$), and total ICSS stimulations/component ($p=0.038$) but not MCRs ($p=0.400$) compared to males.

Figure II.2d-f shows the time course of changes in body weight, mechanical sensitivity, and ICSS performance during and after repeated treatment with vehicle or 2.0 mg/kg/day

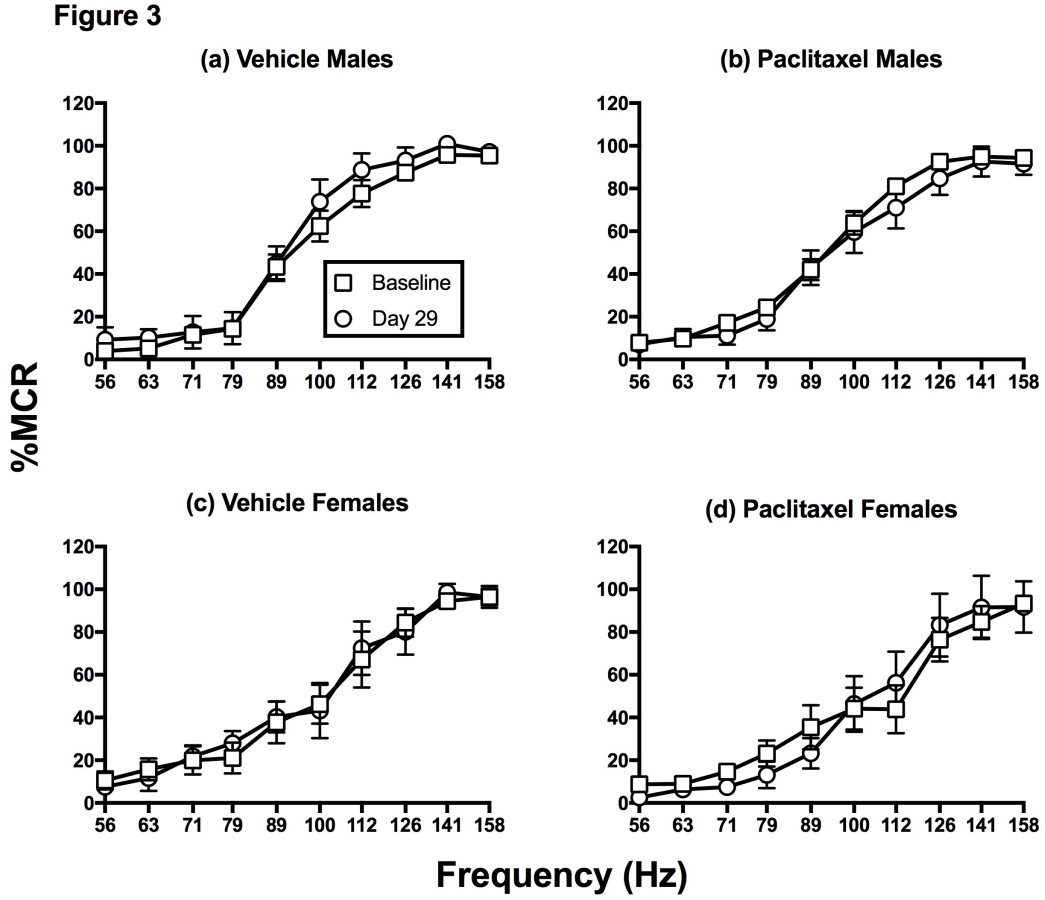

Figure II.3: Comparison of pre-paclitaxel baseline and Day 29 ICSS frequency-rate curves for all rats treated with vehicle or 2.0 mg/kg/day paclitaxel. Panels a-b show the effects of vehicle (N=12) or paclitaxel treatment (N=18) in males, and panels c-d show effects of vehicle (N=6) or paclitaxel (N=6) in females. Horizontal axes: frequency of brain stimulation (Hz). Vertical axes: ICSS rate expressed as percent maximum control rate (%MCR). All points show mean±SEM. Statistical results are as follows. (a) No significant main effect of treatment [$F(1,11)=1.44$; $p=0.255$], a significant effect of frequency [$F(9,99)=118.30$; $p<0.0001$], and no significant interaction [$F(9,99)=0.40$; $p=0.794$]; (b) No significant main effect of treatment [$F(1,17)=0.54$; $p=0.471$], a significant effect of frequency [$F(9,153)=167.4$; $p<0.0001$], and no significant interaction [$F(9,153)=0.44$; $p=0.914$]. (c) No significant main effect of treatment

[F(1,5)=0.01; p=0.930], a significant effect of frequency [F(9,45)=35.78; p<0.0001], and no significant interaction [F(9,45)=0.29; p=0.974]. (d) No significant main effect of treatment [F(1,5)=0.02; p=0.886], a significant effect of frequency [F(9,45)=36.14; p<0.0001], and no significant interaction [F(9,45)=0.89; p=0.541].

paclitaxel. Body weight increased over time in both the vehicle- and the paclitaxel-treated groups, and there was no difference between groups. Mechanical sensitivity did not change in the vehicle-treated group, but relative to the vehicle group, paclitaxel significantly reduced mechanical sensitivity thresholds on Days 8, 15, 22, and 29 following initiation of paclitaxel treatment. Two-way ANOVA indicated that ICSS performance did not change over time in either the vehicle- or paclitaxel-treated groups, and there was no difference in ICSS between groups. Linear regression analysis of ICSS rates over time also indicated no significant difference between the slopes (95%CLs) of the ICSS time-course data after repeated 2.0mg/kg paclitaxel [slope = 0.21 (-0.29, 0.70)] compared to repeated vehicle [slope = 0.45 (0.17, 0.72)]. However, inspection of individual data indicated that ICSS was depressed in one female rat (see below for details).

As a final indication of the weak effects of paclitaxel treatment on ICSS performance in female rats, Figure II.3c-d compares full ICSS frequency-rate curves at baseline and on Day 29 in all females treated with vehicle or 2.0 mg/kg/day paclitaxel. Two-way ANOVA did not indicate a significant main effect of treatment or an interaction between frequency and treatment for either vehicle or paclitaxel.

Lack of correlation between ICSS depression and mechanical hypersensitivity

Figure II.4a shows ICSS data for all male and female rats on the last day of the 29-day study. One-way ANOVA did not reveal a significant effect of paclitaxel dose on ICSS performance, but as noted above, there was substantial individual variability in rats treated with paclitaxel. In particular, 6 of the 24 rats treated with 2.0 mg/kg/day paclitaxel and one of three rats treated with 6.0 mg/kg/day paclitaxel had ICSS rates below those of the lowest saline-treated

Figure 4

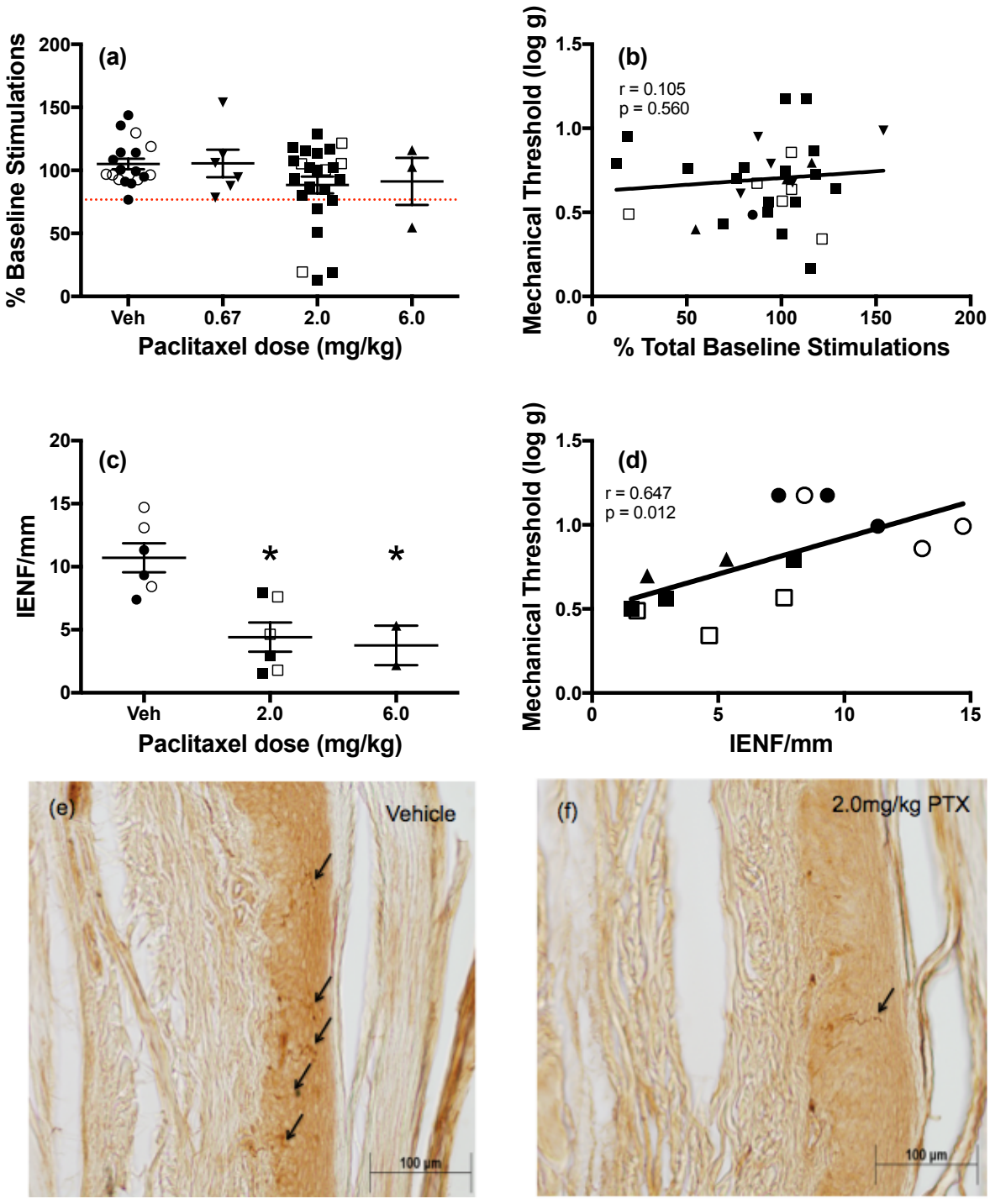
(a)
% Baseline Stimulations
200
150
100
50
0
Veh
0.67
2.0
6.0
Paclitaxel dose (mg/kg)
(b)
r = 0.105
p = 0.560
Mechanical Threshold (log g)
1.5
1.0
0.5
0.0
0
50
100
150
200
% Total Baseline Stimulations
(c)
IENF/mm
20
15
10
5
0
*
*
Veh
2.0
6.0
Paclitaxel dose (mg/kg)
(d)
r = 0.647
p = 0.012
Mechanical Threshold (log g)
1.5
1.0
0.5
0.0
0
5
10
15
IENF/mm
(e)
Vehicle
100 µm
(f)
2.0mg/kg PTX
100 µm

Figure II.4: Paclitaxel effects in individual rats on the last day of the study (Day 29). Closed symbols denote individual male rats and open symbols denote individual female rats. (a) Effects of repeated vehicle (N=18), 0.67 mg/kg (N=6), 2.0 mg/kg (N=24), or 6.0 mg/kg (N=3) paclitaxel on ICSS responding in all male and female rats. Horizontal axis: paclitaxel dose in mg/kg/day. Vertical axis: ICSS performance expressed as % baseline number of brain-stimulation reinforcements earned per 10-min component. Group data shows mean±SEM. One-way ANOVA indicated no significant effect of paclitaxel dose [$F(3,47)=1.52$, $p=0.222$]. Dotted line indicates the lowest value for a vehicle-treated rat, and points below this line suggest paclitaxel-induced ICSS depression in some rats. (b) Correlation of ICSS responding and mechanical sensitivity. Horizontal axis: ICSS performance expressed as % baseline number of brain-stimulation reinforcements earned per 10-min component. Vertical axis: mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g. The correlation was not significant ($r=0.105$; $p=0.560$). (c) Density of Intra-epidermal nerve fibers following repeated vehicle (N=6), 2.0mg/kg (N=6), or 6.0mg/kg (N=2) paclitaxel. Horizontal axis: paclitaxel dose in mg/kg/day. Vertical axis: Density of intra-epidermal nerve fibers (IENF) expressed as fibers per mm. Group data shows mean±SEM. One-way ANOVA indicated a significant effect paclitaxel dose [$F(2,11)=9,30$, $p=0.004$], and asterisks indicate significantly different from vehicle. (d) Correlation of intra-epidermal nerve fiber density and mechanical sensitivity threshold. Horizontal axis: Density of Intra-epidermal nerve fibers (IENF) expressed as fibers per mm. Vertical axis: mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g. The correlation was significant ($r=0.647$; $p=0.012$). (e) Photograph of intra-epidermal nerve fibers in a vehicle-treated rat. (f) Photograph of intra-epidermal nerve fibers in a paclitaxel-treated rat. PTX = paclitaxel. Arrows indicate presence of fibers.

rat. To evaluate the degree to which ICSS depression might be related to magnitude of mechanical hypersensitivity, the ICSS and mechanical sensitivity data from individual paclitaxel-treated rats were submitted to correlational analysis. Figure II.4b shows that magnitude of ICSS depression did not correlate with magnitude of mechanical hypersensitivity in individual rats treated with paclitaxel. However, Figure II.4c shows that intra-epidermal nerve fiber density evaluated in a subset of rats did decrease after paclitaxel treatment, and Figure II.4d shows that intra-epidermal nerve fiber density in these rats did correlate with magnitude of mechanical hypersensitivity. Intra-epidermal nerve fiber density did not correlate with ICSS depression ($r=0.226$; $p=0.438$). Figure II.4e and II.4f depict representative photographs from fiber density analysis

Paclitaxel effects on body weight, mechanical sensitivity and food-maintained responding in male rats

For all male rats used in studies of food-maintained responding, the baseline body weight was 360.2 ± 9.9 g, the baseline mechanical sensitivity threshold was 1.17 ± 0.02 log g, and the baseline rate of food-maintained responding was 135.0 ± 28 reinforcements per session. Figure II.5a-c shows the time course of changes in body weight, mechanical sensitivity, and food-maintained operant responding during and after repeated treatment with vehicle or 2.0 mg/kg/day paclitaxel. For male rats on a restricted diet and with caloric intake dependent on operant responding, repeated 2.0mg/kg paclitaxel caused a significant decrease in body weight from Days 17-29 compared to vehicle treatment. As observed in studies of ICSS operant responding, 2.0 mg/kg/day paclitaxel decreased mechanical sensitivity thresholds compared to vehicle treatment, and this decrease was significant on Days 15 and 29. As with ICSS, two-way ANOVA did not indicate a significant effect of paclitaxel treatment on rates of food-maintained

Figure 5

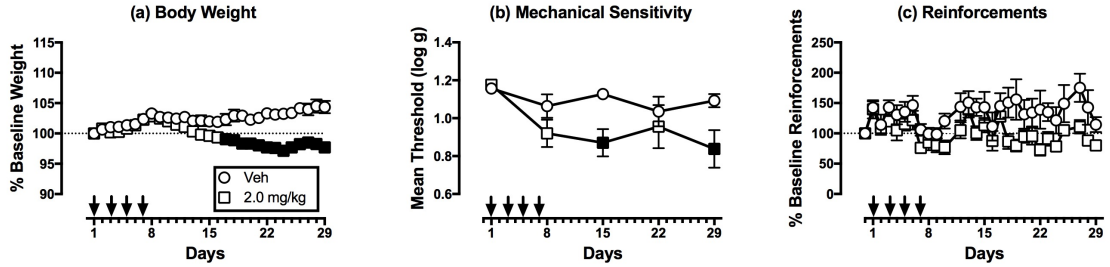

Figure II.5: Paclitaxel effects in male rats responding for food delivery. Horizontal axes: Time in days relative to initiation of vehicle/paclitaxel treatment on Day 1. Arrows indicate vehicle/paclitaxel treatment days. Vertical axes: (a) % baseline body weight, (b) mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g, and (c) operant performance expressed as % baseline number of food pellets earned per 30-min session. All points show mean±SEM for six male rats. Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) Significant main effects of treatment [$F(1,10)=18.17$; $p=0.002$] and time [$F(28,280)=5.83$; $p<0.0001$] and a significant interaction [$F(28,280)=13.69$; $p<0.0001$]. (b) Significant main effects of treatment [$F(1,10)=5.82$; $p=0.037$] and time [$F(5,50)=6.36$; $p=0.0001$], but no significant interaction [$F(5,50)=2.09$; $p=0.082$]. (c) No significant main effect of treatment [$F(1,10)=4.46$; $p=.061$], a significant effect of time [$F(27,270)=3.61$;$p<0.0001$], and no significant interaction [$F(27,270)=1.33$; $p=0.131$].

responding. However, linear regression analysis of food-maintained responding over time indicated that the slope (95%CLs) of the time-course data after repeated 2.0 mg/kg paclitaxel [slope = -0.82 (-1.62 to -0.02)] was significantly different both from zero and from the slope of the data in the vehicle-treated rats [slope = 0.79 (-0.02 to 1.60)].

Figure II.6 shows data from individual rats on the last day of saline or paclitaxel treatment (Day 29). Figure II.6a shows that the 2.0 mg/kg paclitaxel-treated group earned significantly fewer food pellets than the vehicle treated group according to a students t-test ($p=.031$). However, Figure II.6b shows that, for the paclitaxel-treated rats, there was not a significant correlation between depression of food-maintained responding and mechanical hypersensitivity [$r=0.175$, $p=0.745$].

Summary

Chapter II compared effects of paclitaxel treatment on mechanical sensitivity and positively reinforced operant responding in rats. There were three main findings. First, paclitaxel doses sufficient to produce mechanical hypersensitivity and reduce intra-epidermal nerve fiber density in paw skin did not reliably depress ICSS in male or female rats; however, rates of food-maintained responding were modestly decreased in males. Second, analysis of data from individual rats indicated that the degree of behavioral suppression of either ICSS or food-maintained responding did not correlate with mechanical sensitivity. The effectiveness of paclitaxel treatment to decrease operant responding in a subset of rats may be related to paclitaxel-induced functional impairment observed in a subset of human patients. However, the lack of correlation between mechanical sensitivity and behavioral suppression suggests that mechanical hypersensitivity does not cause behavioral suppression, may have different underlying mechanisms than behavioral suppression, and may not serve as a useful surrogate

measure for clinically relevant signs of behavioral depression in neuropathic pain. Third, food-maintained responding was more sensitive to detect chemotherapy-induced depression of motivated behavior than ICSS.

Figure 6

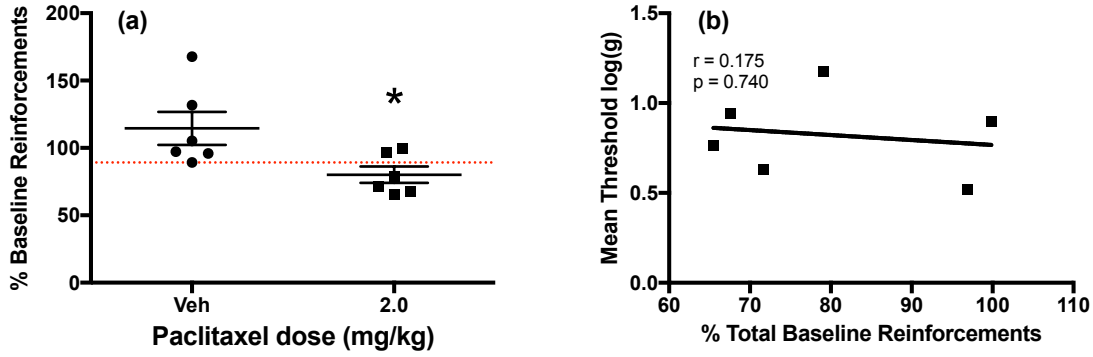

Figure II.6: Paclitaxel effects in individual rats on the last day of the study (Day 29). (a) Effects of repeated vehicle (N=6) or 2.0 mg/kg paclitaxel (N=6) on individual rats' performance in food-maintained responding. Horizontal axis: paclitaxel dose in mg/kg/day. Vertical axis: total number of reinforcements delivered in 30 min test session, expressed as a percentage of the pre-paclitaxel baselines. Group data show mean±SEM. T-test indicated a significant effect of paclitaxel [t(10)=2.52; p=0.031]. Asterisk denotes significance compared to vehicle. Dotted line indicates the lowest value for a vehicle-treated rat, and points below this line suggest paclitaxel-induced depression of food-maintained responding in some rats. (b) Correlation of performance in food-maintained responding and mechanical sensitivity. Horizontal axis: total number of reinforcements delivered in 30 min test session, expressed as a percentage of pre-paclitaxel baselines. Vertical axis: mechanical sensitivity expressed as threshold stimulation to elicit withdrawal in log g. The correlation was not significant (r=0.175; p=0.740).

Chapter III: Differential Effects of Paclitaxel, Vincristine, Oxaliplatin, and Bortezomib on Neuropathic Endpoints

Introduction:

The goal of the present study was to test the hypothesis that paclitaxel, vincristine, oxaliplatin, or bortezomib treatment regiments sufficient to produce mechanical hypersensitivity in rats would also produce depression of operant responding maintained by food delivery in an assay of food-maintained responding. All four chemotherapeutics are known to produce both peripheral neuropathy and chemotherapy-induced neuropathic pain (CINP) in human patients. The dosing regimens implemented for paclitaxel and oxaliplatin have previously produced mechanical hypersensitivity in rodents (Boyette-Davis and Dougherty, 2011; Boyette-Davis et al., 2011a; Toma et al., 2017; Legakis et al., 2018) while different dosing regimens achieving comparable cumulative doses of vincristine and bortezomib have produced mechanical hypersensitivity. Cumulative doses of paclitaxel and oxaliplatin lower than those tested in this study have failed to produce behavioral depression in rodents (Mustafa et al., 2013; Abd-Elsayed et al., 2015; Ling et al., 2017) while no previous studies have investigated the effects of vincristine or bortezomib on operant behavior. Behavioral economic principles were utilized in analysis of chemotherapy effects on operant responding to provide greater resolution of potential behavioral depression effects in terms of reinforcing efficacy of food pellets (Hursh and Winger, 1995; Hursh and Silberberg, 2008). Morphine, an opioid analgesic used with considerable frequency but marginal effectiveness to treat CINP (Eisenberg et al., 2005; Dworkin et al., 2010), was evaluated for its effectiveness to reverse chemotherapy-induced mechanical allodynia and behavioral depression. Nortriptyline, a tricyclic antidepressant that selectively blocks norepinephrine transporters (Sanchez and Hyttel, 1999) and is also sometimes used to treat CINP

(Dworkin et al., 2010) was also evaluated for its effectiveness to reverse chemotherapy-induced behavior depression. Disruptions to proprioception and/or motor output have been observed in patients experiencing chemotherapy-induced neuropathy as evidenced by increased falls (Tofthagen et al., 2012a; Gewandter et al., 2013) and balance impairment (Sarosy et al., 1992; Wampler et al., 2007; Hile et al., 2010; Tofthagen et al., 2012b; Kneis et al., 2016). In addition to evaluation of chemotherapy effects on mechanical sensitivity and food-maintained operant responding, effects were also evaluated on performance of a balance-beam task to assess disruptions to proprioception and/or motor output.

Methods:

Subjects

Studies were conducted in adult male (91) and female (55) Sprague-Dawley rats (Envigo, Somerset, NJ) with initial weights ranging from 356 to 504 g in males and 244 to 320g in females. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM in an AAALAC International-accredited housing facility. Males in studies of food-maintained responding had access to 45 mg food pellets (BioServ Dustless Precision Pellets, Flemington, NJ) during operant behavior sessions, and they were given access to unlimited water and 8.5 ± 0.5 g per day of standard chow diet (Teklad standard diet - 19% protein) one hour after experimental sessions. For all other rats, food and water were available ad libitum in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Drugs

Paclitaxel was obtained as a clinically available 6.0 mg/ml solution (Cardinal Health, Richmond, Virginia,) and diluted in vehicle (8.3% ethanol, 8.3% Cremophor EL, and 83.4% saline) to final a concentration of 2 mg/ml. Vincristine was obtained as a clinically available 1.0 mg/ml solution (Cardinal Health, Richmond, Virginia,) and diluted in saline to final concentrations of 0.0625, 0.125, and 0.25 mg/ml. Oxaliplatin was obtained as a clinically available 5.0 mg/ml solution (Cardinal Health, Richmond, Virginia,) and diluted in saline to final concentrations of 1.25, 2.5, and 5.0 mg/ml. Bortezomib (LC Labs, Woburn, Massachusetts) was dissolved in 5.0% DMSO and saline to final concentrations of 0.0625, 0.125, and 0.25 mg/ml. The "vehicle" for all experiments was a composite vehicle composed of the reagents required to dilute or dissolve the various chemotherapies and was composed of 5.0% DMSO, 5.0% glucose, 8.3% ethanol, and 8.3% Cremophor EL in saline (73.4%). All rats were injected intraperitoneally (i.p.) on four alternate days (Days 1, 3, 5, and 7) with vehicle, paclitaxel (2.0 mg/kg), vincristine (0.0625, 0.125, 0.25 mg/kg), oxaliplatin (1.25, 2.5, 5.0 mg/kg), or bortezomib (0.0625, 0.125, 0.25 mg/kg) using an injection volume of 1 ml/kg. This dosing regimen resulted in cumulative doses of 8.0 mg/kg of paclitaxel, 0.25, 0.50, and 1.0 mg/kg of vincristine, 5.0, 10.0, and 20.0 mg/kg of oxaliplatin, and 0.25, 0.50, and 1.0 mg/kg of bortezomib. Table III.1 shows the experimental timeline for chemotherapy injections and data collection. The dose range for each chemotherapy was determined based on published studies (Amoateng et al., 2015; Fujita et al., 2015; Yamamoto et al., 2015; Legakis et al., 2018) and on preliminary pilot studies that evaluated doses of each chemotherapy that could be administered with little or no lethality using the designated dosing regimen of four injections administered on alternate days (see Results below). Morphine sulfate (National Institute on Drug Abuse Drug Supply Program) and

nortriptyline HCl (Sigma-Aldrich, St. Louis, Missouri) were dissolved in sterile water and administered subcutaneously (s.c.; morphine) or i.p. (nortriptyline) in a volume of 1.0 ml/mg. Doses are expressed in terms of the salt forms listed above.

Pilot studies

Pilot studies were conducted to examine viability of rats during treatment with probe doses of each chemotherapeutic (vincristine: 0.125, 0.25, 0.5 mg/kg/day; oxaliplatin: 2.5, 5.0, 10.0 mg/kg/day; bortezomib: 0.5, 1.0, 2.0 mg/kg/day). Each dose was tested in two rats (one male, one female) using a regimen of four doses administered i.p. on alternating days, and body weights were monitored for 29 days including the first day of treatment. Additionally, paclitaxel was tested by slow (60 sec) intravenous (i.v.) infusion to permit comparison of toxicity by i.v. and previously used i.p. routes of administration. In accordance with moribundity criteria established by the VCU IACUC, rats were euthanized if they lost >20% of their body weight. Doses tested during pilot studies were selected based on published studies with each chemotherapeutic (Amoateng et al., 2015; Fujita et al., 2015; Yamamoto et al., 2015). The primary dependent measures were survival and body weight at conclusion of the 29-day test period.

Experimental Design

Following determination of dose ranges in pilot studies, separate groups of rats were used to study (1) mechanical sensitivity, (2) food-maintained operant responding, or (3) balance-beam performance. The timeline of experimental events for each group is shown in Table III.1.

Table III.1: Timeline of experimental events

Day	Mechanical Sensitivity Experiment	Food-Maintained Responding Experiment	Balance Beam Experiment
-2 - 0	Predrug mechanical sensitivity threshold determination	Predrug total reinforcements determination	Predrug trials on large, medium, and small beams
1, 3, 5, 7	Administration of vehicle, paclitaxel, vincristine, oxaliplatin or bortezomib	Administration of vehicle, paclitaxel, vincristine, oxaliplatin or bortezomib	Administration of vehicle, paclitaxel, vincristine, oxaliplatin or bortezomib
1 - 29	Weekly mechanical sensitivity threshold testing on days 1, 8, 15, 22, and 29; Daily body weight determination	Daily operant testing; Daily body weight determination	Weekly balance beam testing on days 1, 8, 15, 22, and 29
29	Morphine Testing	24-hour sucrose preference test	Not Applicable
30-31	Not Applicable	Mechanical sensitivity threshold testing (30); Adjustment to FR1 operant testing (31)	Not Applicable
32-42	Not Applicable	Demand curve determination (FR 1, 3, 10, 18, 32, 56, 100, 180, 320, 560, 1000)	Not Applicable
43	Not Applicable	Adjustment to FR5 operant testing	Not Applicable
44-53	Not Applicable	Saline (44), morphine (45-46), and nortriptyline (47-53) testing	Not Applicable

Mechanical sensitivity testing with von Frey filaments

Testing procedure. Effects of treatment with vehicle or with each dose of each chemotherapy were evaluated in separate groups of six rats (three male and three female). On test days, rats were placed on elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to acclimate for at least 20 minutes before exposure to mechanical stimuli. Von Frey filaments (ranging from 0.4 to 15.0 g and increasing ~0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar surface of each hindpaw, and the threshold stimulus to elicit paw withdrawal was determined in log grams using the "up-down" method as previously described (Chaplan et al., 1994; Legakis et al., 2018; Legakis and Negus, 2018). Filament forces greater than 15.0 g were not used because they physically lifted the paw, and as a result, paw movement could not be reliably attributed to a withdrawal response by the subject. Briefly, baseline mechanical sensitivity thresholds were determined on the day before initiation of vehicle, paclitaxel, vincristine, oxaliplatin, or bortezomib treatment, and threshold were subsequently redetermined on Days 1, 8, 15, 22 and 29 after initiation of chemotherapy treatment. Mechanical sensitivity thresholds were also determined on Day 30 for rats in the assay of food-maintained operant responding (Table III.1)

Cumulative morphine testing. Following threshold determinations on Day 29, morphine antinociception was evaluated using a cumulative-dosing procedure. Saline and a sequential series of morphine doses (0.32, 0.68, 2.2, 6.8 mg/kg) were administered subcutaneously (s.c.) at 60 min intervals. Each dose increased the total, cumulative morphine dose by 0.5 log units (saline, 0.32, 1.0, 3.2, 10 mg/kg), and mechanical sensitivity thresholds were determined 30 min after each injection.

Data analysis. For each test condition, data were averaged across paws within a rat and then across rats. Effects of each chemotherapy treatment on mechanical sensitivity were analyzed by two-way ANOVA, with time after initiation of treatment as a within-subjects factor and chemotherapy dose as a between subjects factor. A significant ANOVA was followed by Holm-Sidak's post hoc test to compare effects of vehicle with effects of each chemotherapy dose at a given time point. Day 30 mechanical sensitivity thresholds in rats from the assay of food-maintained responding were analyzed by one-way ANOVA followed by a Dunnett's post hoc test to compare effects of vehicle with effects of each chemotherapy. For all analyses here and below, statistical analysis was conducted using Prism 7.0 (Graphpad Software Inc., San Diego, CA), and the criterion for significance was $p<0.05$.

Morphine effects on Day 29 were expressed as Percent Maximum Possible Effect (%MPE) using the equation: %MPE = [(Test – Daily Baseline) ÷ (Ceiling – Daily Baseline)] x 100, where "Test" was the threshold determined after a morphine dose, "Daily Baseline" was the threshold determined before any injection on Day 29, and "Ceiling" was the maximum force tested (15 g). Morphine effects on chemotherapy-induced mechanical hypersensitivity were analyzed by two-way ANOVA with morphine dose as a within-subjects factor and chemotherapy treatment as a between-subjects factor. A significant ANOVA was followed by Holm-Sidak's post hoct test. Additionally, morphine ED50 values and 95% confidence limits were determined by linear regression of data from the linear portion of each morphine dose-effect curve. ED50 values were considered to be significantly different if 95% confidence intervals did not overlap.

Food-maintained operant responding

Apparatus. Studies were conducted in sound-attenuating boxes containing modular acrylic and metal test chambers (29.2 x 30.5 x 24.1 cm; Med Associates, St Albans, VT). Each

chamber contained a response lever, three stimulus lights (red, yellow, and green) centered above the lever, a 2-W house light, and a pellet dispenser that delivered 45 mg food pellets (BioServ Dustless Precision Pellets, Product# F0042, Flemington, NJ) to an aperture beside the lever. Control of stimulus delivery in the operant chamber and collection of data on lever presses and reinforcements earned were accomplished with a computer, interface, and custom software (Med PC-IV, Med Associates).

Training. Effects of treatment with vehicle or a selected dose of each chemotherapy were tested in separate groups of six male rats. Studies of food-maintained operant responding were conducted in male rats as previous studies demonstrated paclitaxel-induced depression of responding for food pellets (Chapter II) and because previous studies found no sex differences in paclitaxel effects on either mechanical hypersensitivity or positively maintained operant responding maintained by electrical brain stimulation (Legakis et al., 2018; Legakis and Negus, 2018). Onset of the house light signaled the beginning of 30-min behavioral sessions under a fixed-ratio (FR) schedule of reinforcement. The FR was gradually increased from FR 1 to FR 5, and after each pellet delivery, there was 0.5-sec time out period during which the lever lights were illuminated and responding had no scheduled consequences. Training continued until the following criteria for stable responding were met for three consecutive days: (1) subjects earned $\geq$ 75 reinforcements/session, and (2) the number of reinforcements/session on each day varied by $\leq$ 5% of the running mean.

Testing. Once responding stabilized on the FR 5 schedule, a 29-day testing protocol began. Thirty-minute operant behavioral sessions were conducted daily (with occasional exceptions on weekends) throughout the 29-day test period, and vehicle or a chemotherapy dose was administered 2 hr before behavioral sessions on Days 1, 3, 5, and 7. For each group of six

rats, the highest tolerated dose (≤ 20% lethality) was selected based on pilot studies and results of the mechanical sensitivity experiments described above (2.0 mg/kg paclitaxel, 0.25 mg/kg vincristine, 5.0 mg/kg oxaliplatin, 0.25 mg/kg bortezomib). All rats were weighed before each operant behavioral session. In addition, mechanical sensitivity was assessed before behavioral testing on Day 30 (methods described above) (Table III.1).

Sucrose preference test. Following behavioral experiments on Day 29, rats were exposed to a 24-hr, two-bottle choice assay in their home cages. One bottle was filled with water and the other was filled with 2% sucrose dissolved in water. Bottles were weighed before and after the 24-hr session, and the change in weight for each bottle was calculated in grams. Data are expressed as percentage of 2% sucrose choice using the equation: % Sucrose Preference = [Sucrose grams ÷ (Sucrose grams + Water grams)] x 100.

Mechanical sensitivity testing. On Day 30, mechanical sensitivity thresholds were determined in all rats as described above.

Demand curve testing. On Days 31 and 32, food pellets were made available under an FR 1 schedule as described above. On each subsequent day, (Days 33-42), the FR was increased using the following progression: 3, 10, 18, 32, 56, 100, 180, 320, 560, and 1000. Operant sessions lasted 30 min, and the FR progression continued in each rat until no pellets were earned.

Morphine and nortriptyline testing. On Day 43, vehicle- and paclitaxel-treated rats were returned to the original FR 5 schedule. On Day 44, saline was administered (s.c.) 30 min prior to operant testing, and responding was still depressed in the paclitaxel-treated rats relative to the vehicle-treated rats. On Days 45 and 46, 1.0 mg/kg morphine and 3.2 mg/kg morphine (s.c.), respectively, were administered 30 min prior to operant testing in vehicle- and paclitaxel-treated rats. Morphine doses were selected based on morphine potency to reverse chemotherapy-induced

mechanical hypersensitivity. On Days 47-53, 3.2 mg/kg nortriptyline (i.p.) was administered 30 min prior to operant testing (Table III.1). The nortriptyline dose was selected based on previous studies of nortriptyline effects on ICSS (Rosenberg et al., 2013).

Data analysis. The primary dependent measure for food-maintained responding was the total number of reinforcements/session. Data from the final three training days prior to initiation of vehicle or chemotherapy treatment were averaged to produce a mean predrug baseline measure of reinforcements/session for each rat. Once vehicle or chemotherapy treatment was initiated, the number of reinforcements/session was determined daily in each rat on Days 1-29 and expressed as a percentage of predrug baseline for each rat using the equation: % Baseline Reinforcements = (Number of Reinforcements on a Test Day ÷ Predrug Baseline Reinforcements) x 100. Changes in food-maintained responding over time were then averaged across rats and analyzed by two-way ANOVA, with time after initiation of treatment as a within-subjects factor and chemotherapy treatment as a between-subjects factor. A significant ANOVA was followed by the Holm-Sidak post-hoc test.

For analysis of data collected on Days 31-41, when FR values were progressively increased, the number of reinforcements per session was plotted as a function of FR value for each chemotherapeutic. These data were fit using a custom-designed GraphPad Prism template (freely available from the Institutes for Behavior Resources, *http://www.ibrinc.org*) with the Exponential Model of Demand (Hursh and Silberberg, 2008) using the equation $Log\ Q = Log\ Q_0 + k\ (e^{-a * Q0 * C} - 1)$ where Q represents reinforcers earned, Q_0 represents theoretical number of reinforcers earned if the response requirement were zero, k represents log10 value of the greatest number of observed reinforcers earned, e represents the base of the natural logarithm, α represents a free parameter that is adjusted to minimize the difference between predictions of the

equation and each demand curve, and *C* represents the response requirement (i.e., FR). When analyzing demand functions of individual subjects, data were included up to the highest FR value at which at least one reinforcer was earned. When analyzing aggregate demand functions within a chemotherapeutic group, a subject's zero value would be included in the analysis if it could be averaged with another subject's non-zero number of reinforcers at a given FR value. The scaling variable κ was fixed to a shared value of 2.56 when analyzing individual demand functions or 2.46 when analyzing aggregate demand functions, as these numbers correspond to the log10 value of the greatest number of reinforcements earned at any response requirement by any single animal or by any group during treatment with a single chemotherapy, respectively. Demand elasticity (α) and free consumption (Q_0) values were determined from individual demand functions and compared between chemotherapies using one-way ANOVA tests.

Data from the sucrose preference test and mechanical sensitivity testing were also compared between chemotherapies using one-way ANOVA.

Balance beam assessment

Apparatus. The apparatus consisted of an open starting platform (20 cm x 20 cm) and an enclosed goal box (20 cm x 20 cm x 20 cm) connected by a removable beam with a flat surface that the rat had to cross to go from starting platform to goal box. Three different beam widths were used (0.75, 0.5, and 0.375 inches). The starting platform, beam, and goal box were elevated 50 cm above the base and bench top.

Training procedure. Rats were initially trained using the widest beam width (0.75 inches). During a training trial, the rat was placed on the starting platform and given a maximum of 20 sec to cross the beam to the goal box once placing both paws on the beam. Training continued with up to three trials per day until rats crossed the beam without falling in ≤20

seconds on at least six consecutive trials. Rats that failed to meet this criterion were excluded from further testing (1 male rat).

Testing procedure. Once training was complete, effects of vehicle or doses of each chemotherapy that were tested in food-maintained responding experiments (see above) were evaluated in a separate group of 34 rats (17 males and 17 females). A baseline test session was conducted on Day 0, before chemotherapy treatment, and on Days 8, 15, 22, and 29 following initiation of treatment. Each test session consisted of six trials, with two trials at each of the three beam widths. Beam widths were presented in order of widest to thinnest. The primary dependent measure was beam-cross time, defined as the time between leaving the starting platform (both forepaws leaving the starting platform and making contact with the beam) and time to enter the goal box (both hindpaws entering the goal box). On each trial, the maximum time allowed to initiate a beam cross was 10 min, and the maximum time allowed to complete a cross once initiated was 20 sec. If a rat failed to complete a cross before these cutoff times, then a beam-cross time of 20 sec was assigned. No rats failed to initiate a beam cross under the allotted 10 minutes. Trials were separated by at least 30 min, and both trials on each beam were completed in one rat before proceeding to the next rat.

Data analysis. Test sessions were filmed, and endpoints were quantified using Windows Media Player. Data were analyzed by two-way ANOVA, with time after initiation of treatment as a within-subjects factor and chemotherapy drug as a between-subjects factor. A significant ANOVA was followed by Holm-Sidak's post hoc test to compare effects of vehicle with effects of each chemotherapy dose at a given time point.

Results:

Chemotherapy effects in pilot studies

Table III.2 shows results of the pilot dose-ranging study. Doses that produced either > 20% body weight loss or death in at least one of the two tested were deemed to be too high for inclusion in the main study. On the basis of these data, the maximum tolerable dose of each chemotherapeutic for subsequent studies was 0.25 mg/kg for vincristine, 5.0 mg/kg for oxaliplatin, and 0.25 mg/kg for bortezomib. The maximum tolerable dose of paclitaxel was determined previously (Legakis et al., 2018), and the present pilot study suggested that toxicity associated with i.v. paclitaxel administration was similar to that of i.p. administration.

Table III.2: Summary of pilot dosing studies to determine safety the following chemotherapy drugs: vincristine, oxaliplatin, bortezomib, and (I.V.) paclitaxel.

Drug and Dose	**Male** Lethal or <80% Body Weight	**Female** Lethal or <80% Body Weight
0.125 mg/kg Vincristine	No	No
0.25 mg/kg Vincristine	No	No
0.5 mg/kg Vincristine	Yes	Yes
2.5 mg/kg Oxaliplatin	No	No
5.0 mg/kg Oxaliplatin	No	No
10.0 mg/kg Oxaliplatin	Yes	Yes
0.5 mg/kg Bortezomib	No	Yes
1.0 mg/kg Bortezomib	Yes	Yes
2.0 mg/kg Bortezomib	Yes	Yes
6.0 mg/kg Paclitaxel (I.V.)	Yes, Yes (N=2)	Not tested

Chemotherapy effects in rats evaluated for mechanical sensitivity

For rats used in studies of mechanical sensitivity, the baseline body weight means ± SEM were 394.2 ± 6.3 g (males) and 278.4 ± 3.3 g (females) and the baseline mechanical-sensitivity thresholds means ± SEM were 1.17 ± 0.00 log g (males) and 1.16 ± 0.01 g (females). Figure III.1 shows the time course of changes in body weight during and after repeated treatment with vehicle and each chemotherapy in rats also tested for mechanical sensitivity. Body weight increased over time in vehicle-treated rats. Body weights in rats treated with paclitaxel or bortezomib did not differ from vehicle-treated controls, whereas vincristine and oxaliplatin dose-dependently decreased body weights relative to vehicle-treated controls.

Figure III.2 shows the time course of changes in mechanical-sensitivity thresholds during and after repeated treatment with vehicle and each chemotherapy. As reported previously in a more extensive dose-effect study (Legakis et al., 2018), 2.0 mg/kg paclitaxel produced mechanical hypersensitivity relative to vehicle-treated controls. Similarly, vincristine, oxaliplatin, and bortezomib also produced dose-dependent mechanical hypersensitivity. The emergence of mechanical hypersensitivity was time-dependent for all chemotherapies, with peak effects observed at the end of the 29-day observation period.

Figure III.3 shows morphine effects on mechanical sensitivity thresholds on Day 29 after treatment with the highest dose of each chemotherapy. Results are shown as raw thresholds in log g in Figure III.3a and as %MPE in Figure III.3b. Morphine dose-dependently reversed mechanical hypersensitivity produced by all four chemotherapies, and in each case, a dose of 3.2 mg/kg was the lowest dose to produce significant effects. Table III.3 shows the calculated morphine ED50 values based on %MPE for each of the chemotherapy doses that were sufficient to produce significant hypersensitivity on Day 29. Despite the varying magnitudes of

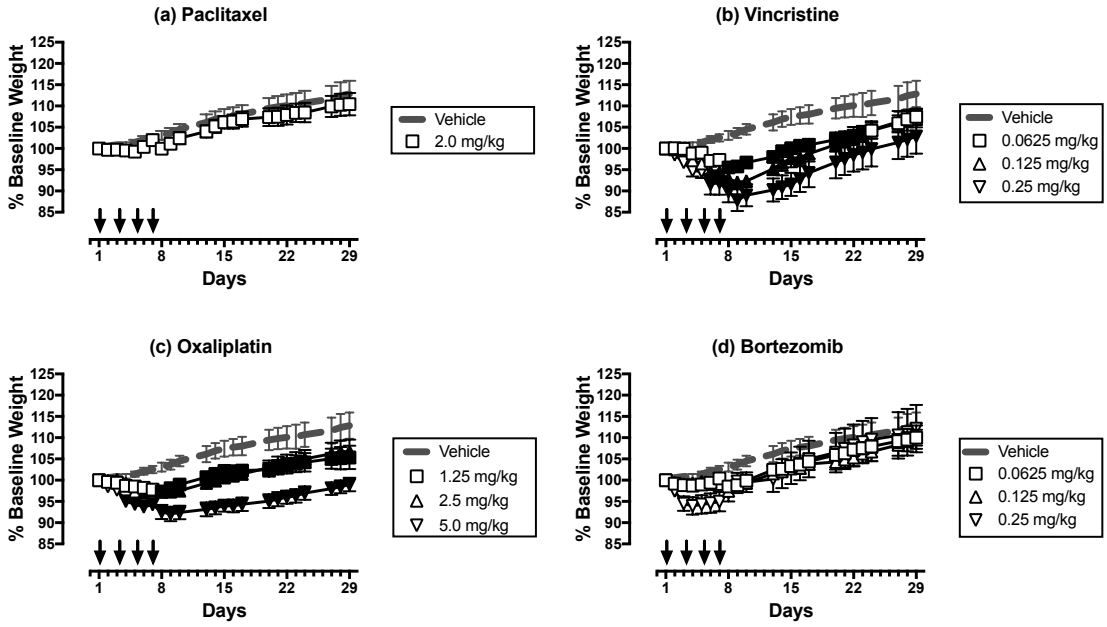

Figure III.1: Effects of paclitaxel (a), vincristine (b), oxaliplatin (c), and bortezomib (d) on body weight of free-feeding rats also tested for mechanical sensitivity. Horizontal axes: Time in days relative to initiation of vehicle or chemotherapy treatment on Day 1. Arrows indicate treatment days. Vertical axes: body weight expressed as % Baseline Weight determined before initiation of treatment. All points show mean±SEM for N=6 rats (3 male and 3 female). Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) No significant main effect of treatment [$F(1,10)=0.66$; $p=0.437$], a significant effect of time [$F(24,240)=1520$; $p<0.0001$], and no significant interaction [$F(24,240)=0.31$; $p=0.999$]. (b) Significant main effects of treatment [$F(3,20)=6.91$; $p=0.002$] and time [$F(22,440)=40.74$; $p<0.0001$] and a significant interaction [$F(66,440)=2.75$; $p<0.0001$]. (c) Significant main effects of treatment [$F(3,20)=11.13$; $p<0.001$] and time [$F(22,440)=36.20$; $p<0.0001$] and a significant interaction [$F(66,440)=4.12$; $p<0.0001$]. (d) No significant main effect of treatment

[F(3,20)=0.88; p=0.467], a significant effect of time [F(22,440)=46.99; p<0.0001], and no significant interaction [F(66,440)=1.66; p=0.189].

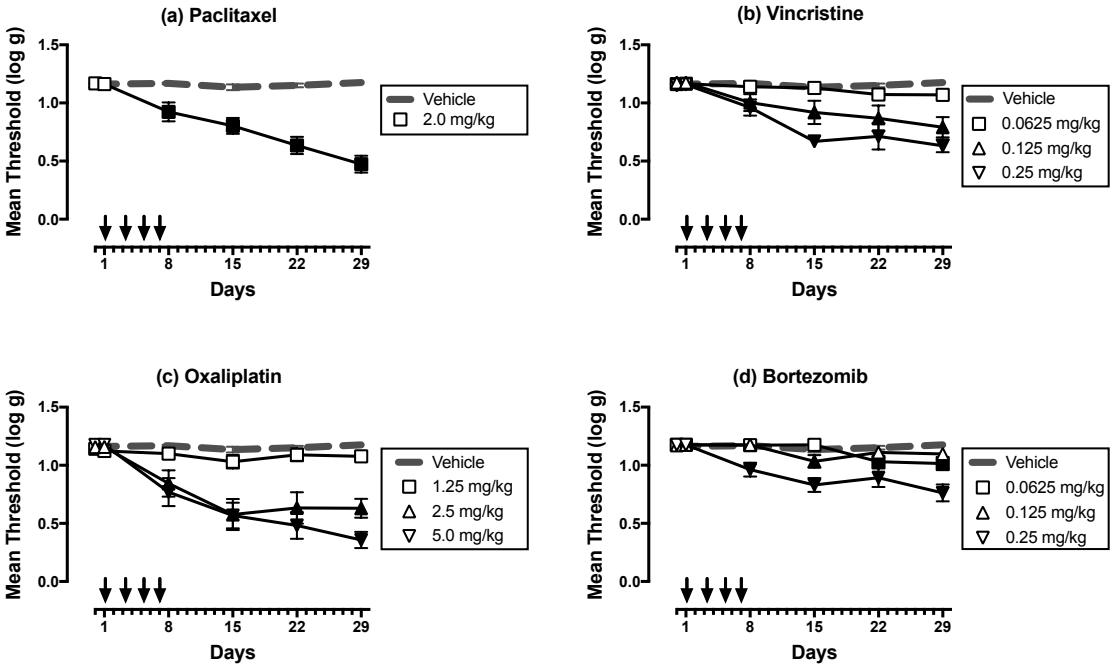

Figure III.2: Dose-dependent effects of paclitaxel (a), vincristine (b), oxaliplatin (c), and bortezomib (d) on mechanical sensitivity. Horizontal axes: Time in days relative to initiation of vehicle or chemotherapy treatment on Day 1. Arrows indicate treatment days. Vertical axes: mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g. All points show mean±SEM for N=6 rats (3 male and 3 female). Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) Significant main effects of treatment [$F(1,10)=56.15$; $p<0.0001$] and time [$F(5,50)=26.74$; $p<0.0001$], and a significant interaction [$F(5,50)=27.83$; $p<0.0001$]. (b) Significant main effects of treatment [$F(3,20)=14.57$; $p<0.0001$] and time [$F(5,100)=23.37$; $p<0.0001$], and a significant interaction [$F(15,100)=6.21$; $p<0.0001$]. (c) Significant main effects of treatment [$F(3,20)=18.26$; $p<0.0001$] and time [$F(5,100)=34.07$; $p<0.0001$], and a significant interaction [$F(15,100)=9.99$; $p<0.0001$].

(d) Significant main effects of treatment [$F(3,20)=14.98$; $p<0.0001$] and time [$F(5,100)=19.28$; $p<0.0001$], and a significant interaction [$F(15,100)=7.18$; $p<0.0001$].

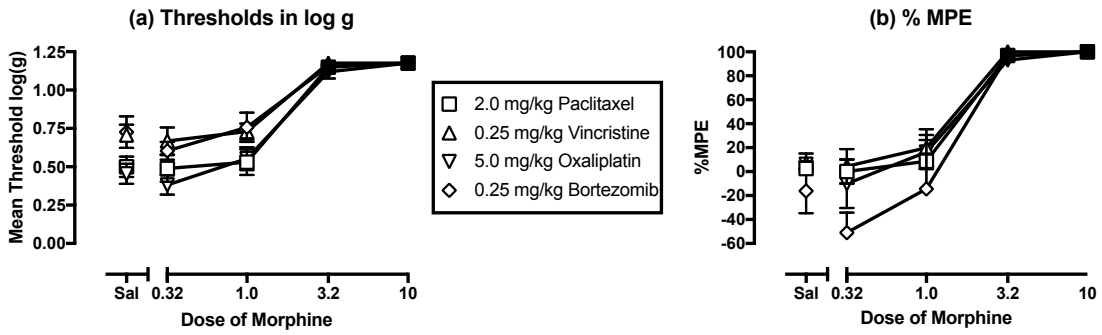

Figure III.3: Effects of morphine on mechanical hypersensitivity induced by maximum tolerated doses of paclitaxel, vincristine, oxaliplatin, and bortezomib. Horizontal axes: Cumulative dose of morphine in mg/kg (log scale). Vertical axes: mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g (a) or morphine effects expressed as percent maximal possible effect (%MPE) (b). All points show mean±SEM for N=6 rats (3 male and 3 female). Filled points indicate a significant difference from saline (Sal) at a given dose as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows: (a) Significant main effects of treatment [$F(3,20)=3.68$; $p=0.029$] and morphine dose [$F(4,80)=158.6$; $p<0.0001$], and no significant interaction [$F(12,80)=1.87$; $p=0.051$]. (b) No significant main effect of treatment [$F(3,20)=1.85$; $p=0.171$], a significant effect of morphine dose [$F(4,80)=78.04$; $p<0.0001$], and no significant interaction [$F(12,80)=0.757$; $p=0.692$].

Table III.3: Morphine ED50 values to reverse chemotherapy-induced mechanical hypersensitivity on Day 29.

Drug and Dose	ED50 in mg/kg (95%Confidence Limits)
2.0 mg/kg Paclitaxel	1.35 (1.01 - 1.83)
0.125 mg/kg Vincristine	1.03 (0.33 -3.24)
0.25 mg/kg Vincristine	1.24 (0.60 - 2.56)
2.5 mg/kg Oxaliplatin	0.85 (0.08, 8.66)
5.0 mg/kg Oxaliplatin	1.470 (0.44, 4.89)
0.0625 mg/kg Bortezomib	2.54 (1.52, 4.22)
0.25 mg/kg Bortezomib	1.01 (0.32- 3.20)

chemotherapy-induced hypersensitivity, there were no differences in morphine potency to reverse mechanical hypersensitivity.

Chemotherapy effects in rats evaluated for food-maintained responding

For male rats used in studies of food-maintained responding, the baseline mean ± S.E.M. body weight was 338.4 ± 3.2 and the predrug baseline mean ± S.E.M. rate of reinforcements per session was 173.9 ± 7.6. Figure III.4 shows the effects of treatment with vehicle or the maximum dose of paclitaxel (2.0 mg/kg), vincristine (0.25 mg/kg), oxaliplatin (5.0 mg/kg), and bortezomib (0.25 mg/kg) that was evaluated in the mechanical-sensitivity studies described above. Relative to the stable rates of reinforcement over time in vehicle-treated rats, paclitaxel produced small but significant decreases in rates of reinforcement on Days 4, 8, 10, 22, and 24-29. Vincristine oxaliplatin, and bortezomib each produced significant decreases in rates of reinforcement at various times during the first two weeks of observation, but with these

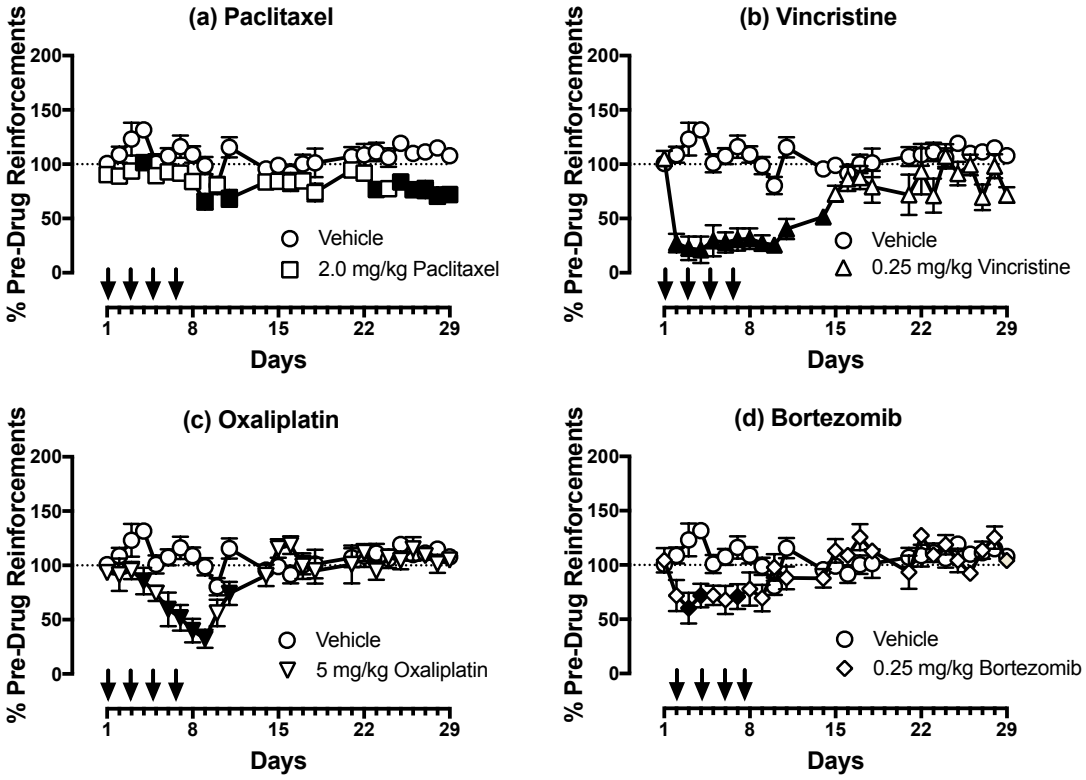

Figure III.4: Effects of paclitaxel (a), vincristine (b), oxaliplatin (c), and bortezomib (d) on rates of reinforcement in an assay of food-maintained operant responding. Horizontal axes: Time in days relative to initiation of vehicle or chemotherapy treatment on Day 1. Arrows indicate treatment days. Vertical axes: reinforcements earned per session expressed as %Pre-Drug Reinforcements per session prior to vehicle/chemotherapy administration. All points show mean±SEM for N=6 male rats. Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) Significant main effects of treatment [$F(1,10)=23.21$; $p<0.001$] and time [$F(24,240)=3.149$; $p<0.0001$], and a significant interaction [$F(24,240)=1.90$; $p=0.008$]. (b) Significant main effects of treatment [$F(1,10)=46.31$; $p<0.0001$]

and time [$F(24,240)=5.98$; $p<0.0001$], and a significant interaction [$F(24,240)=6.85$; $p<0.0001$]. (c) No significant main effect of treatment [$F(1,10)=4.95$; $p=0.050$], but a significant effect of time [$F(24,240)=6.67$; $p<0.0001$], and a significant interaction [$F(24,240)=5.82$; $p<0.0001$]. (d) No significant main effect of treatment [$F(1,10)=2.87$; $p=0.121$], but a significant effect of time [$F(24,240)=3.12$; $p<0.0001$], and a significant interaction [$F(24,240)=4.7$; $p<0.0001$].

chemotherapies, rates of reinforcement were not different from those in vehicle-treated rats during the last two weeks.

Figure III.5 shows changes in body weights over the same time period in these rats. For these studies, food access was limited, and body weights increased only slightly in vehicle-treated rats. Relative to vehicle controls, body weights were not altered in rats treated with paclitaxel, oxaliplatin, or bortezomib; however, vincristine significantly decreased body weights from Days 5-29.

Figure III.6 shows vehicle and chemotherapy effects on sucrose preference (Days 29-30) and mechanical sensitivity (Day 30). Sucrose preference did not differ across groups, but mechanical sensitivity thresholds were decreased in paclitaxel-, vincristine-, and oxaliplatin-treated rats compared to vehicle-treated rats. Bortezomib did not significantly reduce mechanical hypersensitivity on Day 30 for rats in the assay of food-maintained responding

As a final assessment of chemotherapy effects on food reinforcement, Figure III.7 shows demand curves for food pellets determined in each group of rats during Days 32-42. Increasing ratio requirements decreased the number of pellets earned in all groups, and there was no difference across groups in parameters for either maximum consumption at the lowest FR value (Q_0) or in elasticity of demand with increasing FR value (α).

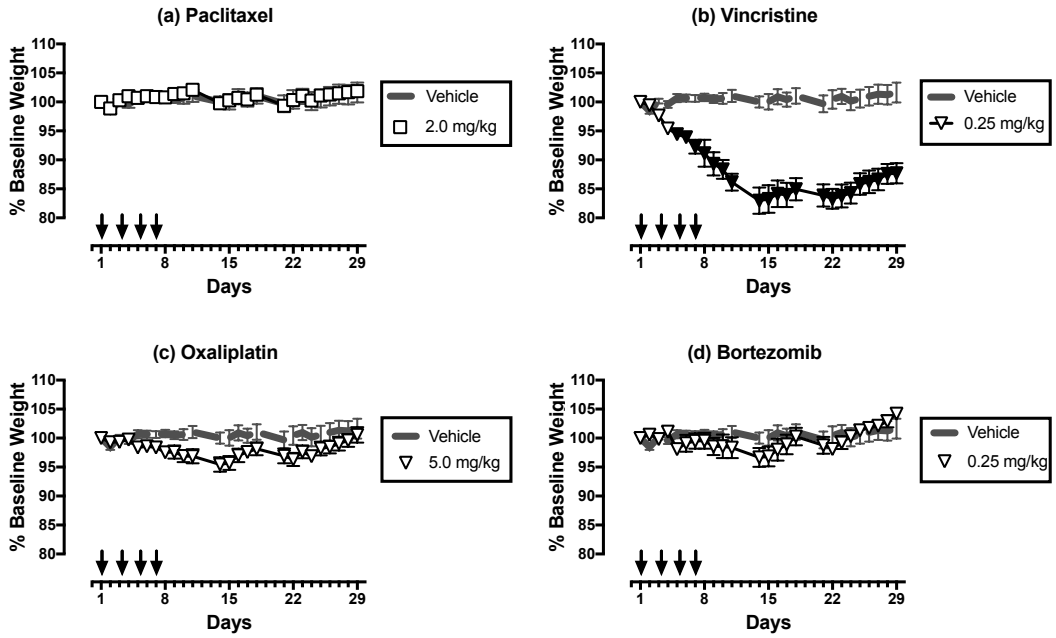

Figure III.5: Dose-dependent effects of paclitaxel (a), vincristine (b), oxaliplatin (c), and bortezomib (d) on body weight of food-restricted male rats in the assay of food-maintained operant responding. Horizontal axes: Time in days relative to initiation of vehicle or chemotherapy treatment on Day 1. Arrows indicate treatment days. Vertical axes: body weight expressed as % Baseline Weight determined before initiation of treatment. All points show mean±SEM for N=6 male rats. Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) No significant main effect of treatment [$F(1,10)=0.06$; $p=0.816$], a significant effect of time [$F(24,240)=3.04$; $p<0.0001$], and no significant interaction [$F(24,240)=0.27$; $p>0.999$]. (b) Significant main effects of treatment [$F(1,10)=47.64$; $p<0.0001$] and time [$F(24,440)=17.90$; $p<0.0001$] and a significant interaction [$F(24,240)=21.87$; $p<0.0001$]. (c) No significant main effect of treatment [$F(1,10)=3.53$; $p=0.090$] but a significant effect of time [$F(24,240)=3.15$; $p<0.0001$] and a significant interaction

[F(24,240)=2.70; p<0.0001]. (d) No significant main effect of treatment [F(1,10)=0.31; p=0.590] but a significant effect of time [F(24,240)=4.11; p<0.0001], and a significant interaction [F(24,240)=2.74; p<0.0001].

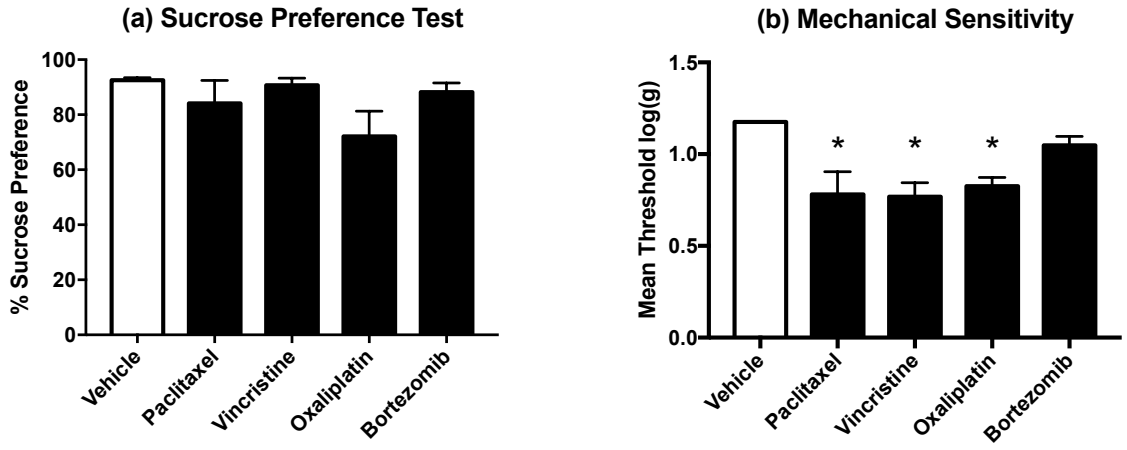

Figure III.6: Effects of vehicle or chemotherapy treatment on sucrose preference (a) and mechanical sensitivity (b) in rats also tested for food-maintained operant responding. Horizontal axis: Treatment delivered on Days 1, 3, 5, and 7. Vertical axis: % Sucrose Preference determined on Days 29-30 after treatment initiation (a) or mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log g (b). All bars show mean±SEM for N=6 male rats. Asterisks (*) denote significant difference as determined by Dunnett's post hoc test after a significant one-way ANOVA, $p<0.05$ from vehicle. Statistical results are as follows: (a) No significant effect of chemotherapy treatments [$F(4,25)=1.93$; $p=0.138$], (b) Significant effect of chemotherapy treatments [$F(4,25)=6.46$; $p=0.001$].

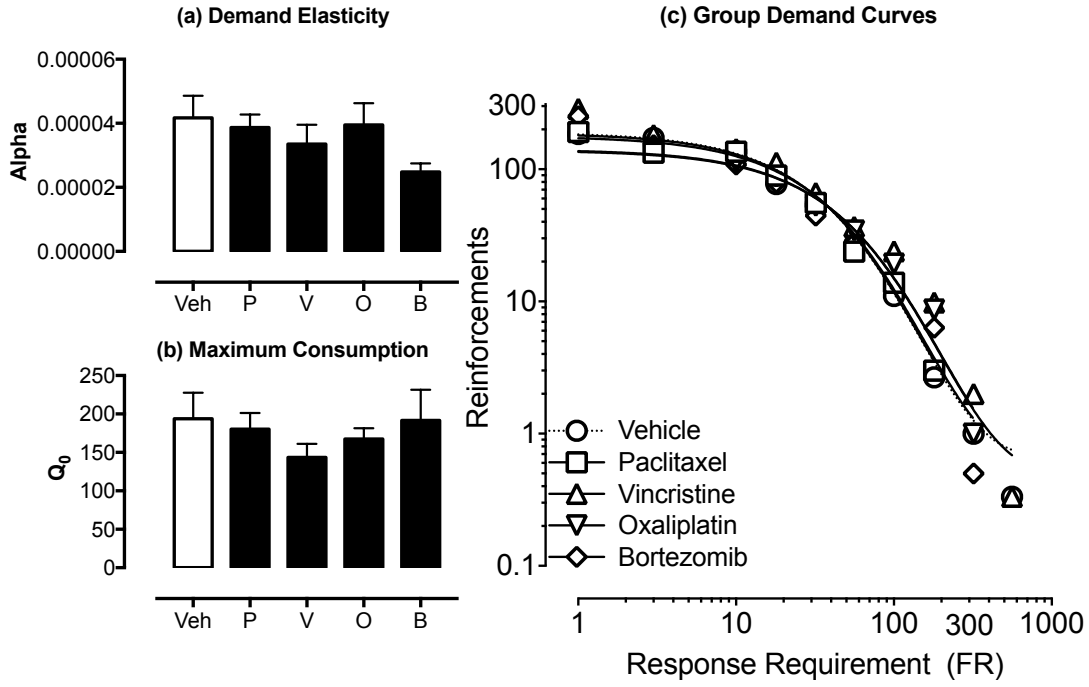

Figure III.7: Effects of vehicle or chemotherapy on parameters of demand for food pellets. Panels III.7a and III.7b show the effects of vehicle (Veh), paclitaxel (P), vincristine (V), oxaliplatin (O), and bortezomib (B) on demand elasticity alpha (a) and maximum consumption Q_0 (b). Panel III.7c shows the summary of aggregated essential values across chemotherapeutics. Statistical results are as follows: (a) No significant effect of chemotherapy treatments [$F(4,25)=1.47$; $p=0.241$], (b) No significant effect of chemotherapy treatments [$F(4,25)=0.90$; $p=0.477$].

Following data collection for demand curves, the FR value was returned to FR 5, and rates of reinforcement were again significantly lower in paclitaxel- than in vehicle-treated rats (data not shown). To evaluate the degree to which lower reinforcement rates might be related to chemotherapy-induced neuropathic pain, the vehicle- and paclitaxel-treated rats were pretreated with saline, 1.0, and 3.2 mg/kg morphine before sessions of food-maintained responding on Days 44-46. Figure III.8a shows that reinforcement rates were significantly lower in paclitaxel-treated rats than in vehicle-treated rats after saline pretreatment. Morphine failed to increase reinforcement rates in chemotherapy-treated rats. Rather, 1.0 mg/kg morphine significantly decreased reinforcement rate in vehicle-treated rats but not in paclitaxel-treated rats while also eliminating the differences between groups. The higher dose of 3.2 mg/kg morphine significantly decreased reinforcements earned in both vehicle- and paclitaxel-treated rats, while again eliminating the differences between groups.

Figure III.8b also shows the effects of repeated treatment with 3.2 mg/kg nortriptyline on Days 47-53. As with morphine, nortriptyline failed to increase rates of reinforcement in paclitaxel-treated rats. Rather, nortriptyline significantly decreased reinforcements earned for all seven days in vehicle-treated rats, for the first day in paclitaxel-treated rats, and eliminated the difference between the groups across all seven days.

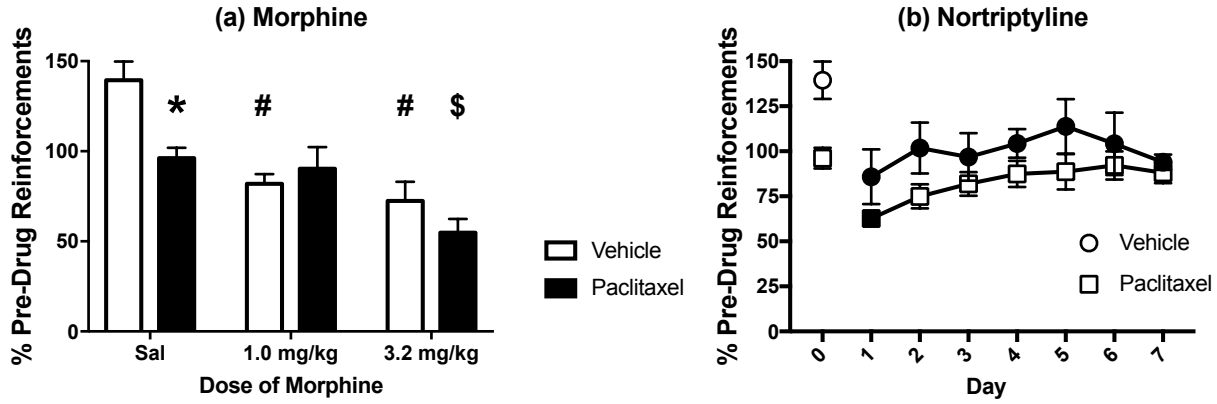

Figure III.8: Effects of morphine (a) and nortriptyline (b) on food-maintained operant responding in rats treated previously with vehicle or paclitaxel. Horizontal axis: Dose of morphine in mg/kg (a) or days of treatment with 3.2 mg/kg nortriptyline (b). Vertical axis: reinforcements earned expressed as %Pre-Drug Reinforcements earned prior to vehicle/chemotherapy administration. All bars show mean±SEM for N=6 male rats. In Panel a, asterisk (*) indicates significant difference between vehicle- and paclitaxel-treated rats with saline administration, pound (#) indicates significant difference between morphine dose and saline in vehicle-treated rats, and dollar ($) indicates significant difference between morphine dose and saline in paclitaxel-treated rats as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. In Panel b, filled points indicate a significant difference from saline (Day 0) on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Statistical results are as follows. (a) Significant main effect of paclitaxel treatment [$F(2,20)=5.20$; $p-=0.046$], a significant effect of morphine dose [$F(2,20)=19.37$; $p<0.0001$], and a significant interaction [$F(2,20)=4.37$; $p=0.027$]. (b) No significant main effect of paclitaxel treatment [$F(1,10)=4.49$; $p=0.060$], a significant effect of nortriptyline treatment day [$F(7,70)=4.74$; $p=0.0002$], and no significant interaction [$F(7,70)=1.00$; $p=0.443$]

Chemotherapy effects in rats evaluated for balance-beam performance

For rats used in studies of balance-beam performance, the baseline mean ± S.E.M, baseline beam-cross times per trial were 2.81 ± 0.25, 5.03 ± 0.46, and 8.29 ± 0.76 sec for the large, medium, and small beams, respectively. These baseline times per trial were significantly different from each other in a beam width-dependent manner by one-way ANOVA. Figure III.9 shows the effects of treatment with vehicle or the same doses of paclitaxel (2.0 mg/kg), vincristine (0.25 mg/kg), oxaliplatin (5.0 mg/kg), and bortezomib (0.25 mg/kg) used in studies of food-maintained responding. In general, the chemotherapies increased beam-cross times, but the expression of this impairment varied across chemotherapies. Paclitaxel increased cross times for the medium beam on Days 15 and 22. Vincristine increased cross times on the large and medium beams on Day 8, and oxaliplatin increased cross-time only on the large beam on Day 15. Bortezomib failed to alter cross time at any time on any beam.

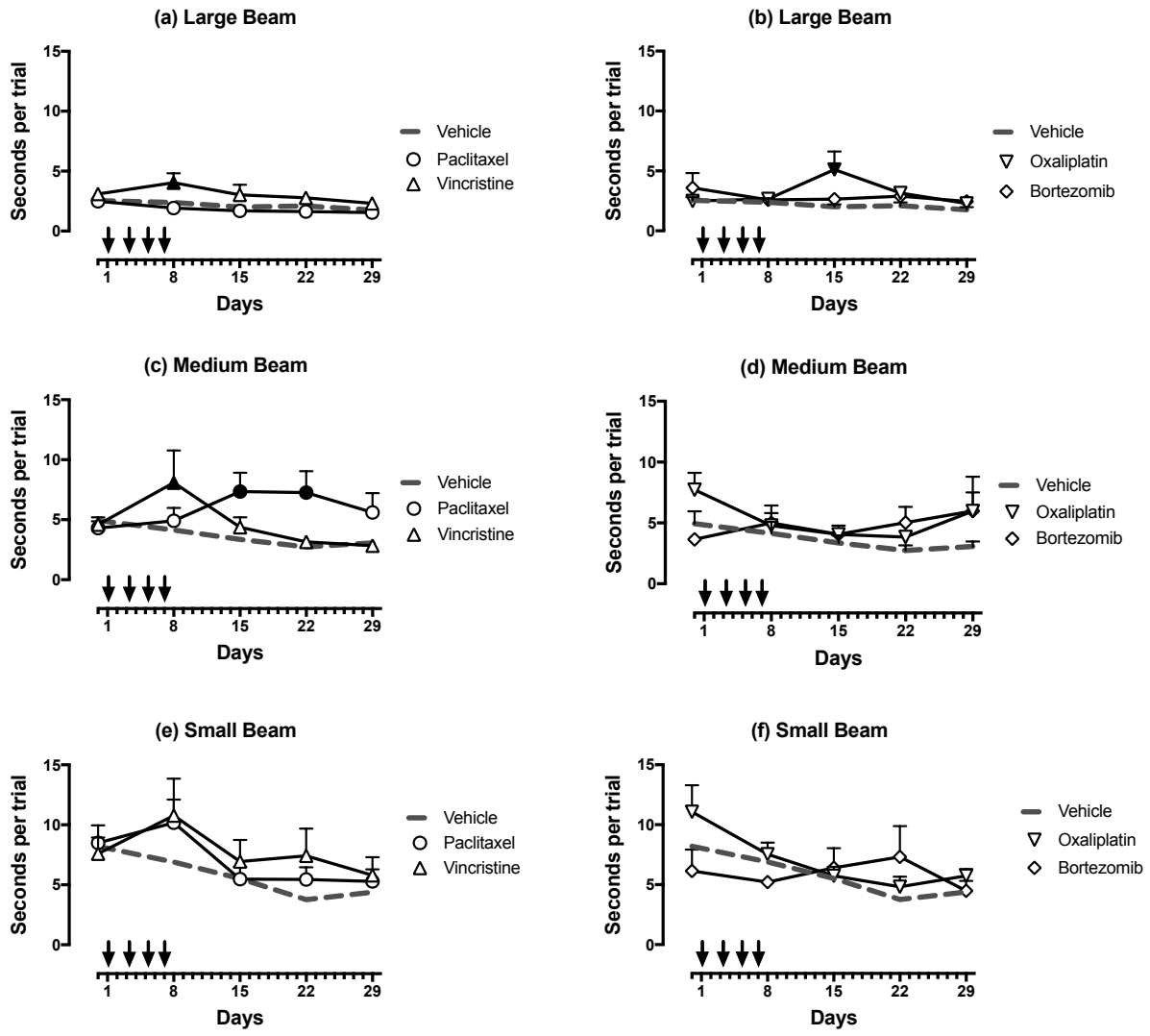

Figure III.9: Effects of vehicle, paclitaxel, vincristine, oxaliplatin, and bortezomib on time to cross balance beams. Panels a and b show the effects of vehicle (a,b), 2.0 mg/kg paclitaxel (a), 0.25 mg/kg vincristine (a), 5.0 mg/kg oxaliplatin (b), and 0.25 mg/kg bortezomib (b) on time to cross the large beam (3/4 inch) per trial. Panels c and d show the effects of vehicle (c,d), paclitaxel (c), vincristine (c), oxaliplatin (d), and bortezomib (d) on time to cross the medium beam (1/2 inch) per trial. Panels e and f show the effects of vehicle (e,f), paclitaxel (e), vincristine (e), oxaliplatin (f), and bortezomib (f) on time to cross the small beam (3/8) per trial.

Horizontal axes: Time in days relative to initiation of vehicle or chemotherapy treatment on Day 1. Arrows indicate treatment days. Vertical axes: time to cross given beam per trial in seconds. All points show mean±SEM for N=10 (5 male, 5 female) vehicle-treated rats and N=6 (3 male, 3 female) for paclitaxel-, vincristine-, oxaliplatin-, and bortezomib-treated rats. Filled points indicate a significant difference from vehicle on a given day as indicated by the Holm-Sidak post hoc test after a significant two-way ANOVA, $p<0.05$. Drugs are separated in the graph above for clarity of presentation, but for statistical analysis, data for all treatments (vehicle and each chemotherapy) for a given beam width were evaluated in a single two-way ANOVA. Statistical results are as follows: (a,b) Significant main effects of treatment [$F(4,29)=4.08$; $p=0.010$] and time [$F(4,116)=2.87$; $p=0.026$], and a significant interaction [$F(16,116)=1.95$; $p=0.023$]. (c,d) No significant main effects of treatment [$F(4,29)=1.39$; $p=0.263$], no effect of time [$F(4,116)=0.72$; $p=0.579$], but a significant interaction [$F(16,116)=2.25$; $p=0.007$]. (e,f) No significant main effects of treatment [$F(4,29)=0.77$; $p=0.551$], a significant effect of time [$F(4,116)=5.57$; $p=0.0004$], and no significant interaction [$F(16,116)=1.10$; $p=0.37$]

Summary

Chapter III compared the effects of four neuropathic classes of chemotherapeutics using paclitaxel, vincristine, oxaliplatin, and bortezomib on mechanical hypersensitivity and motivated behavior. There were three main findings. First, differential effects were observed with the four different chemotherapeutics. All chemotherapies produced dose-dependent and sustained mechanical hypersensitivity, although the magnitude of that hypersensitivity was greater for paclitaxel and oxaliplatin than vincristine and bortezomib. Similarly, all chemotherapies significantly decreased food-maintained responding, though only paclitaxel produced a sustained yet weak depression weeks after initiation of treatment. Finally, all chemotherapies except bortezomib significantly impaired balance beam performance, with paclitaxel once again inducing greater magnitude and longer duration of effects. Second, despite the small decrease in responding maintained by food delivery under the FR 5 schedule observed 29 days after paclitaxel treatment, none of the chemotherapies significantly altered either sucrose preference or demand for food pellets assessed on Days 31-43 after initiation of treatment. Thus, even for paclitaxel, effects on food-maintained responding were weak and observed only at later time points with only one "cost" of food reward. Third, morphine was effective to reverse paclitaxel-, vincristine-, oxaliplatin-, and bortezomib-induced mechanical hypersensitivity but was unable to reverse paclitaxel-induced behavioral depression of food-maintained responding.

Chapter IV: Repeated Morphine Produces Sensitization to Reward and Tolerance to Anti-allodynia in Male and Female Rats with Chemotherapy-Induced Neuropathy

(Published in Journal of Pharmacology and Experimental Therapeutics, in press, PMID: 29363579)

Introduction:

The goal of the present study was to evaluate the impact of a chronic pain state on the emergence of opioid reward that occurs with repeated opioid exposure. Chemotherapy-induced neuropathic pain (CINP) is a common and dose-limiting side effect in the use of chemotherapeutic agents like paclitaxel for cancer treatment (Reeves et al., 2012; Speck et al., 2013; Seretny et al., 2014), and opioid agonists are commonly used to treat CINP (Plante and VanItallie, 2010), despite evidence for marginal therapeutic efficacy (Raja et al., 2002; McNicol et al., 2013; Argyriou et al., 2014). Iatrogenic opioid addiction is well documented in patients with CINP (Ballantyne and LaForge, 2007; Anghelescu et al., 2013; Koyyalagunta et al., 2013; Barclay et al., 2014; Del Fabbro, 2014; Rauenzahn et al., 2017), but it is not clear if CINP alters opioid abuse liability or merely provides an occasion for opioid exposure. Accordingly, the present study examined effects of repeated morphine administration on ICSS in rats treated with paclitaxel or its vehicle. Effects of repeated morphine on ICSS were compared to effects of the same regimen of repeated morphine treatment on paclitaxel-induced mechanical allodynia (Polomano et al., 2001; Pascual et al., 2010; Boyette-Davis et al., 2011a; Hwang et al., 2012; Ko et al., 2014). Studies were conducted in male and female rats because sex differences have been reported previously for some paclitaxel effects (Naji-Esfahani et al., 2016) and for the rewarding and antinociceptive effects of morphine (Cicero et al., 2003; Lynch, 2006; Craft, 2008; Lynch et al., 2013).

Methods:

Subjects

Studies were conducted in adult male and female Sprague-Dawley rats. At the start of the study, males weighed 362 to 488g, and females weighed 265 to 324g. Rats were housed individually and maintained on a 12-h light/dark cycle with lights on from 6:00AM to 6:00PM in an AAALAC International-accredited housing facility. Food and water were available ad libitum in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Academy of Science's Guide for the Care and Use of Laboratory Animals.

Drugs and Experimental Design

Figure IV.1 shows the overall experimental design. For studies of both mechanical sensitivity and intracranial self-stimulation (ICSS), pre-paclitaxel baseline behavioral measures were determined in each rat before a 29-day protocol of treatment and testing. On Days 1, 3, 5, and 7, all rats were treated with either paclitaxel (2.0 mg/kg/day; total paclitaxel dose of 8.0 mg/kg) or vehicle. Paclitaxel was obtained as a clinically available 6.0 mg/ml solution (Cardinal Health, Richmond, Virginia, USA) and diluted in vehicle (8.3% ethanol, 8.3% Cremophor EL, and 83.4% saline) to a final concentration of 1.0 mg/ml for intraperitoneal (i.p.) administration in volume of 2.0 ml/kg. On Days 22 and 29, morphine was administered using a cumulative dosing regimen for determination of morphine dose-effect curves. In this regimen, a sequence of five injections was administered at 50 min intervals, and each successive injection increased the total, cumulative morphine dose by 0.5 log units (0, 0.32, 1.0, 3.2, and 10 mg/kg). On intervening days 23-28, subjects were treated with a single injection of either 3.2 mg/kg/day morphine or

Figure 1

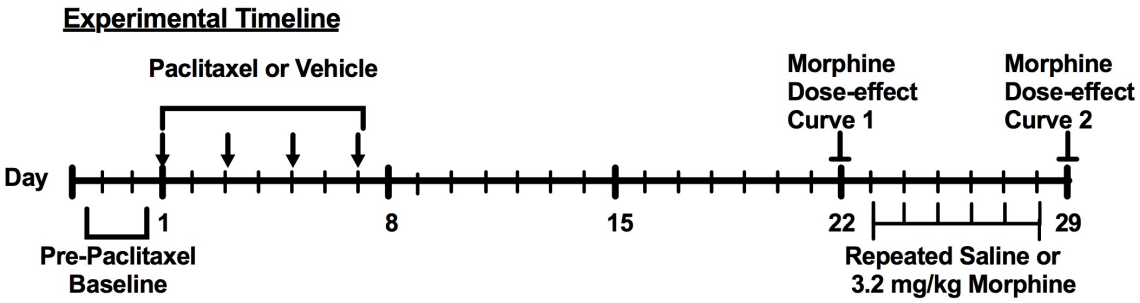

Figure IV.1: Overview of the experimental timeline for treatment and data collection. Baseline measurements were collected on Day 0 (for mechanical sensitivity) and Day -2 to Day 0 (ICSS). 2.0 mg/kg/day paclitaxel or vehicle was injected (i.p.) on Days 1, 3, 5, and 7. Mechanical sensitivity threshold was tested weekly, and ICSS responding was tested daily. Cumulative morphine dose-effect testing was conducted on Days 22 and 29, and repeated treatment with either 3.2 mg/kg/day morphine or saline was administered on intervening Days 23-28.

vehicle. The morphine dose for repeated treatment was selected because it was the lowest dose to block paclitaxel-induced mechanical hypersensitivity (see Results). Morphine sulfate (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) was dissolved in sterile water and administered subcutaneously (s.c.) in a volume of 1.0 ml/kg. Morphine doses are expressed in terms of the sulfate salt.

Mechanical sensitivity testing with von Frey filaments

Testing procedure. To evaluate mechanical sensitivity, rats were first placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to acclimate for at least 20 min before exposure to the mechanical stimuli. Subsequently, von Frey filaments (ranging from 0.4 to 15.0 g and increasing in ~0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar surface of each hindpaw, and the threshold stimulus to elicit paw withdrawal was determined in log grams using the "up-down" method as previously described (Chaplan et al., 1994; Leitl et al., 2014b). Filament forces greater than 15.0 g were not used because they physically lifted the paw, and as a result, paw movement could not be reliably attributed to a withdrawal response by the subject.

The goal of the study was to establish paclitaxel-induced mechanical hypersensitivity, and then to evaluate the impact of repeated morphine treatment on the dose-effect curve for morphine reversal of hypersensitivity. Baseline mechanical sensitivity thresholds were determined on the day before initiation of paclitaxel treatment. All rats in this phase of the study then received paclitaxel. A paclitaxel vehicle control was not included because baseline thresholds in individual rats were generally at the 15.0 g ceiling of the assay, pilot studies indicated no change in baseline after vehicle treatment, and it was not possible to detect morphine-induced increases in thresholds from this high baseline. Following initiation of

paclitaxel treatment, thresholds were reassessed weekly on Days 8, 15, 22, and (if hypersensitivity criterion was met) 29. Specifically, rats that met a criterion level of paclitaxel-induced mechanical hypersensitivity (mean threshold in log grams < 0.90; 15 of 18 males, 17 of 19 females) were subdivided by sex into two cohorts to receive either repeated 3.2 mg/kg/day morphine (N=8 males, 9 females) or repeated saline (N=7 males, 8 females) on Days 23-28. All of these rats also received cumulative morphine (0-10 mg/kg) on Days 22 and 29 to determine morphine dose-effect curves before and after the repeated treatment regimen. Mechanical sensitivity thresholds were determined beginning 30 min after each injection. Rats that failed to meet the criterion for paclitaxel-induced mechanical hypersensitivity (3 of 18 males, 2 of 19 females) were removed from the study.

Data analysis. For each test condition, data were averaged across paws within a rat and then across rats. Changes in thresholds over time were analyzed by one-way ANOVA, and a significant ANOVA was followed by Dunnett's post hoc test to compare post-paclitaxel thresholds with the pre-paclitaxel baseline. Additionally, the potential for sex differences in paclitaxel effects was evaluated by two-way ANOVA with day as a within-subjects variable and sex as a between-subjects variable. Morphine effects were expressed as Percent Maximum Possible Effect (%MPE) using the equation: %MPE = [(Test – Daily Baseline) ÷ (Ceiling– Daily Baseline)] x 100, where "Test" is the threshold determined after a morphine dose, "Daily Baseline" is the threshold determined before any injection on a given test day, and "Ceiling" is the maximum force tested (15 g). Morphine effects on paclitaxel-induced mechanical hypersensitivity were analyzed by two-way ANOVA with dose and treatment day as the two within-subjects factors. For this and all subsequent two-way ANOVAs, a significant ANOVA was followed by the Holm-Sidak post hoc test. For all analyses, the criterion for significance was

set at $P<0.05$. Additionally, the morphine ED50 value was defined as the morphine dose to produce 50% MPE, and morphine ED50 values and 95% confidence limits were determined by linear regression of data from the linear portion of each morphine dose-effect curve. ED50 values were considered to be significantly different if confidence limits did not overlap.

Intracranial self-stimulation (ICSS)

Surgery. Fourteen male and fourteen female rats were anesthetized with isoflurane (2.5-3% in oxygen; Webster Veterinary, Phoenix, Arizona, USA) and implanted with electrodes (Plastics One, Roanoke, Virginia, USA) in the left medial forebrain bundle at the level of the lateral hypothalamus using previously published procedures and coordinates (Males: 2.8 mm posterior to bregma, 1.7 mm lateral to the midsagittal suture, 8.8 mm below skull surface; Females: 3.8 mm posterior to bregma, 1.6 mm lateral to the midsaggital suture, 8.7 mm below skull surface (Lazenka et al., 2016a; Lazenka et al., 2016b)). The electrode was secured to the skull with orthodontic resin and skull screws. Ketoprofen (Spectrum Chemical, New Brunswick, NJ, 5 mg/kg) was administered immediately and 24 hours after surgery as a postoperative analgesic, and rats recovered for 7 days prior to initiation of ICSS training.

Apparatus. Studies were conducted in sound-attenuating boxes containing modular acrylic and metal test chambers (29.2 x 30.5 x 24.1 cm; Med Associates, St Albans, VT, USA). Each chamber contained a response lever (4.5 cm wide, 2.0 cm deep, 3.0 cm above the floor), three stimulus lights (red, yellow, and green) centered 7.6 cm above the lever, a 2-W house light, and an ICSS stimulator. Electrodes were connected to the stimulator via bipolar cables routed through a swivel commutator (Model SL2C, Plastics One, Roanoke, VA, USA). Computers and interface equipment operated by custom software controlled all operant sessions and data collection (Med PC-IV, Med Associates).

Training. Rats were trained to respond for brain stimulation using procedures identical to those previously described (Altarifi and Negus, 2011; Negus and Miller, 2014; Miller et al., 2015). Briefly, a white house light was illuminated during behavioral sessions, and responding under a fixed-ratio (FR) 1 schedule produced a 500-ms train of 0.1-ms square-wave cathodal pulses together with 500-ms illumination of stimulus lights over the response lever. The terminal schedule consisted of sequential 10-min components. Each component consisted of 10 1-min trials, and the available brain-stimulation frequency decreased in 0.05 log Hz increments from one trial to the next (158-56 Hz). Each frequency trial consisted of a 10-s timeout, during which five noncontingent stimulations were delivered at the frequency available during that trial, followed by a 50-s "response" period, during which responding resulted in electrical stimulation. Training continued with presentation of three sequential components per day until the following two criteria for stable responding were met for three consecutive days: (1) ≤5% variability in the maximum rate of reinforcement in any trial, and (2) ≤10% variability in the total number of stimulations per component.

Testing. Three-component ICSS sessions were conducted daily (with occasional exceptions on weekends) throughout the pre-paclitaxel baseline period and subsequent 29-day test period. The final three days of training were used to establish pre-paclitaxel baseline data. On Days 1, 3, 5, and 7, rats were treated with either 2.0 mg/kg/day paclitaxel (n = 8 females and 8 males) or vehicle (n = 6 females and 6 males), and three-component ICSS sessions began 2 hours following paclitaxel or vehicle injections. Daily ICSS testing continued on Days 8-21 without treatment. Additionally, mechanical sensitivity was assessed using methods described above on Day 22, and all paclitaxel-treated rats met the criterion for paclitaxel-induced mechanical hypersensitivity (mean threshold in log grams < 0.90). Subsequently, all rats

received cumulative morphine (0-10 mg/kg) on Days 22 and 29 and repeated treatment with 3.2 mg/kg/day morphine on Days 23-28. Control studies were not conducted in rats treated with repeated saline on Days 23-28, because we have shown previously that repeated saline treatment under these conditions does not alter morphine dose-effect curves on ICSS (Miller et al., 2015). Rather, the goal of this study was to evaluate the hypothesis that repeated morphine would increase expression of morphine-induced ICSS facilitation in rats treated with paclitaxel vehicle but not in rats treated with paclitaxel. For cumulative-dosing test sessions on Days 22 and 29, three daily-baseline components were followed by a series of five consecutive 50-min morphine-test cycles. Treatment injections were administered at the beginning of each test cycle, and two ICSS test components (lasting a total of 20 min) began 30 min after each injection. For single-dose test sessions conducted on the intervening Days 23-28, three daily-baseline components were followed first by administration of 3.2 mg/kg morphine and then 30-min later by two ICSS test components.

Data analysis. Data were analyzed as previously described (Altarifi and Negus, 2011; Negus and Miller, 2014; Miller et al., 2015). The primary dependent measure was the total number of reinforcements per component (i.e. the total number of stimulations delivered across all brain-stimulation frequencies during each 10-min component). All daily sessions consisted of at least three ICSS components. The first component of each daily session was considered to be a "warm up" component, and data were discarded. Data from the remaining pair of components were averaged within each rat and then across rats, and these data provided a measure of "Daily-Baseline" ICSS performance. In addition, behavioral sessions on Days 22-29 also included pairs of morphine test components (5 pairs on cumulative-dosing Days 22 and 29, 1 pair on single-

dose Days 23-28). Data from each pair of morphine test components were also averaged first within each rat and then across rats.

Paclitaxel effects on baseline ICSS performance and on morphine-induced changes in ICSS were analyzed separately. First, to compare effects of vehicle and paclitaxel treatment on daily-baseline ICSS, the number of daily-baseline stimulations per component on Days 1-29 were expressed as a percentage of the pre-paclitaxel baseline using the equation: % Pre-Paclitaxel Baseline Reinforcements per Component = (Daily-Baseline Reinforcements per Component on a Test Day ÷ Pre-Paclitaxel Baseline Reinforcements per Component) x 100. Data in males and females were analyzed using separate two-way ANOVAs, with treatment day as a within-subjects factor and paclitaxel/vehicle treatment as a between-subjects factor. Second, to evaluate morphine effects on ICSS in vehicle- and paclitaxel-treated rats, morphine-test data on a given day were expressed as a percentage of daily-baseline data on that day using the equation: % Daily-Baseline Reinforcements per Component = (Morphine-Test Reinforcements per Component ÷ Daily-Baseline Reinforcements per Component) x 100. Morphine dose-effect data from cumulative dose-effect curves on Days 22 and 29 were compared using separate repeated-measures two-way ANOVAs in male and female vehicle- and paclitaxel-treated rats, with morphine dose and treatment day as the two within-subjects factors. Morphine single-dose test data on Days 23-28 were analyzed by separate two-way ANOVAs in males and females, with treatment day as a within-subject factor and treatment as a between-subjects factor. Lastly, morphine dose-effect data in male and female rats were directly compared to evaluate potential sex differences in morphine effects on Day 22 and Day 29. For this comparison, data were collapsed across paclitaxel and paclitaxel vehicle treatments for each day and compared by two-

way ANOVA with morphine dose as a within-subjects factor and sex as a between-subjects factor.

A secondary and more granular measure of ICSS performance was the reinforcement rate in stimulations per frequency trial. Raw reinforcement rates for each rat from each trial were converted to percent maximum control rate (%MCR), with MCR defined as the mean of the maximal rates observed at any trial during either the pre-paclitaxel baseline sessions (for analysis of paclitaxel effects) or the daily baseline (for analysis of morphine effects). Thus, %MCR values for each trial were calculated as {(reinforcement rate during a frequency trial ÷ MCR) × 100}. %MCR values were then averaged across rats and analyzed by repeated-measures two-way ANOVA, with ICSS frequency and treatment day as the two within-subjects factors.

Results:

Repeated morphine effects on paclitaxel-induced mechanical hypersensitivity in male and female rats

For the 32 rats that completed the mechanical sensitivity studies, baseline mechanical sensitivity thresholds were 1.18 ± 0.00 log g (males) and 1.12 ± 0.06 log g (females). Figure IV.2a and IV.2b shows that paclitaxel produced significant mechanical hypersensitivity on Days 8, 15, 22, and 29 in both males and females. There were no differences between rats treated on Days 23-28 with repeated 3.2mg/kg/day morphine or vehicle in either sex (data not shown), so the data are collapsed to include both subgroups per sex. Additionally, there was also no sex difference in paclitaxel-induced mechanical hypersensitivity as indicated by two-way ANOVA of data in Figure IV.2a and IV.2b with sex and time as the two factors (no significant effect of sex or sex x time interaction). Figure IV.2c and IV.2d show that morphine dose-dependently reversed paclitaxel-induced hypersensitivity in both males and females before (Day 22) and after

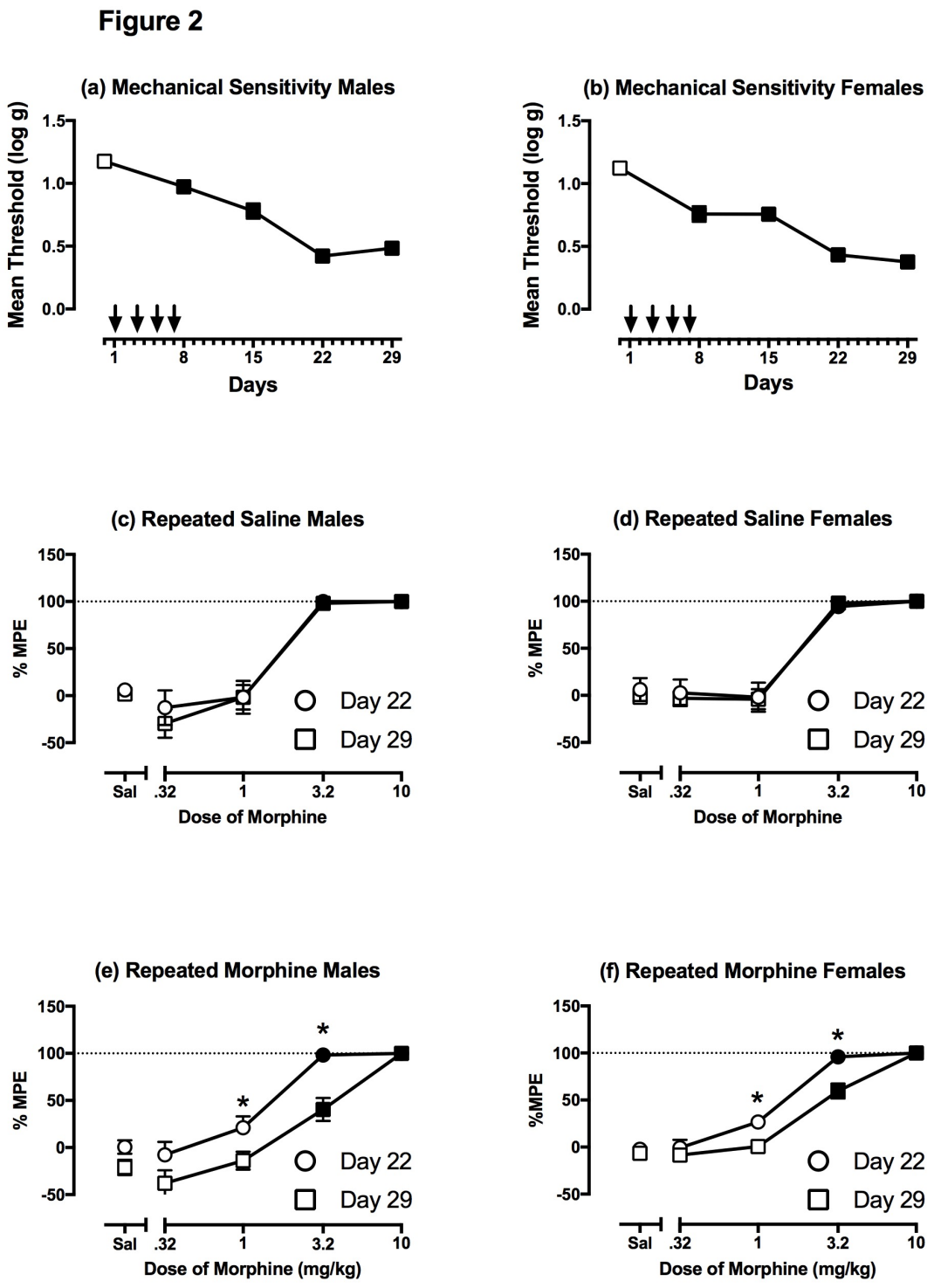

Figure IV.2: Effects of morphine on paclitaxel-induced mechanical hypersensitivity in male and female rats. Panels a-b show the effects of repeated 2.0 mg/kg/day paclitaxel on mechanical sensitivity thresholds in (a) male and (b) female rats. Horizontal axes: Time in days relative to

initiation of vehicle/paclitaxel treatment on Day 1. Arrows show days of paclitaxel treatment. Vertical axes: Mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log grams. All points show mean±SEM (a, N=15; b, N=17), and filled points indicate significantly different from Day 0 pre-paclitaxel baseline. Panels c-f show the effects of morphine on mechanical sensitivity thresholds following paclitaxel treatment. Horizontal axes: Cumulative dose of morphine in mg/kg. Vertical axes: Percent Maximal possible effect (%MPE). All points show mean±SEM (c, N=7; d, N=8; e, N=8; f, N=9), filled points indicate a significant difference from saline (Sal) on a given day, and asterisks denote a significant difference between days at a given morphine dose. Statistical results are as follows: (a) Significant main effect of treatment [$F(3.143, 44)=62.92$; $P<0.0001$]; (b) Significant main effect of treatment [$F(3.087,49.39), = 41.53$; $P<0.0001$]; (c) Significant main effect of treatment [$F(4,24)=51.93$; $P<0.0001$], no significant main effect of day [$F(1,6)=0.5255$; $P=0.496$], and no significant interaction [$F(4,24)=0.46$; $P=0.765$]. (d) Significant main effect of treatment [$F(4,28)=109.2$; $P<0.0001$], no significant effect of day [$F(1,7)=0.77$; $P=0.789$], and no significant interaction [$F(4,28)=0.18$; $P=0.949$]. (e) Significant main effects of treatment [$F(4,28)=65.37$; $P<0.0001$] and day [$F(1,7)=17.27$; $P=0.004$], and a significant interaction [$F(4,28)=5.33$; $P=0.003$]. (f) Significant main effects of treatment [$F(4,32)=135.7$; $P<0.0001$] and day [$F(1,8)=27.00$; $P=0.001$], and a significant interaction [$F(4,32)=4.46$; $P=0.006$].

(Day 29) repeated vehicle treatment on Days 23-28. Two-way ANOVA indicated no difference between morphine dose-effect curves on Day 22 and Day 29 in either sex, and Table IV.1 shows that morphine ED50 values were similar on Day 22 and 29 in both sexes. Figure IV.2e and IV.2f and Table IV.1 show that modest but significant tolerance developed to morphine effects in rats treated with repeated 3.2 mg/kg/day morphine on Days 23-28. Thus, by Day 29, the morphine dose-effect curve had shifted to the right and the morphine ED50 values were significantly increased in both sexes.

Table IV.1. Morphine ED50 values in mg/kg (95% confidence limits) to reverse paclitaxel-induced mechanical hypersensitivity before and after repeated treatment with either saline or 3.2 mg/kg/day morphine on Days 23-28. *Asterisks indicate significantly different from Day 22 as indicated by non-overlapping confidence limits.

Treatment & Test Day	Males	Females
Repeated Saline		
Day 22	1.9 (1.4 – 2.8)	1.8 (1.3 – 2.7)
Day 29	2.2 (1.6 -3.0)	1.9 (1.4 – 2.6)
Repeated Morphine		
Day 22	1.6 (1.1 - 2.3)	1.5 (1.2 - 1.9)
Day 29	3.7 (2.6 -5.4)*	2.6 (2.1 - 3.1)*

Paclitaxel effects on ICSS responding in male and female rats

For rats used in ICSS studies, pre-paclitaxel/vehicle baseline measures of ICSS performance were 137.9 ± 13.9 (males) and 114.8 ± 12.7 (females) stimulations per component and maximum control rates (MCR) were 57.6 ± 5.7 (males) and 53.1 ± 2.4 (females) stimulations per trial. Paclitaxel treatment had little or no effect on ICSS in either sex. Figure IV.3a and IV.3b shows the effects of vehicle and repeated 2.0 mg/kg paclitaxel on the total

Figure 3

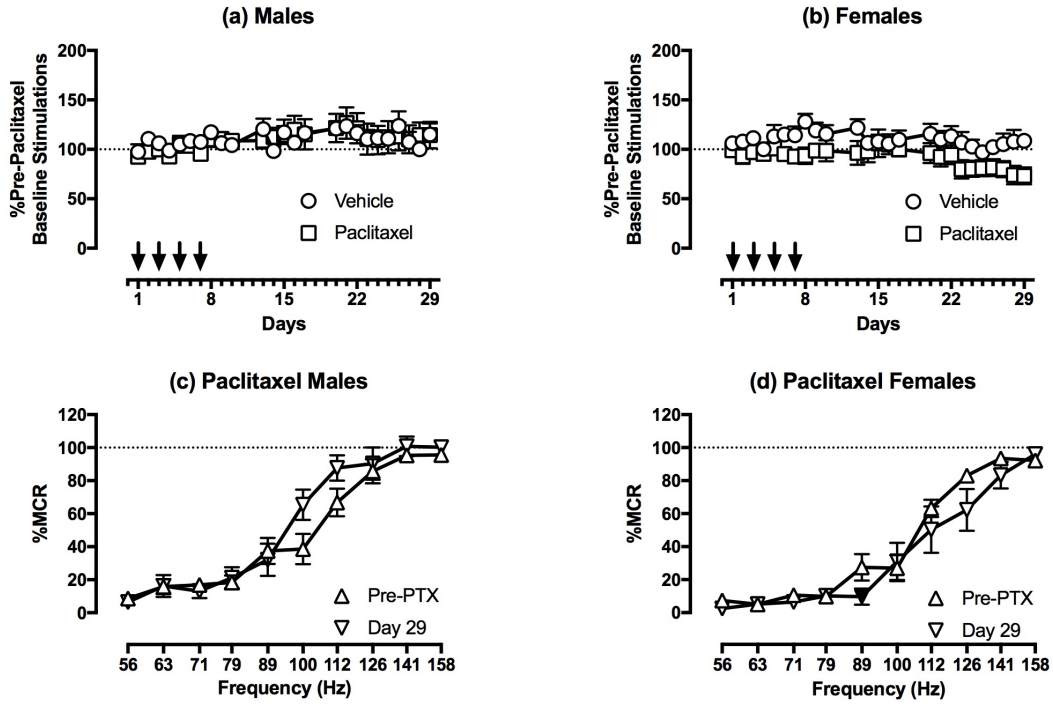

Figure IV.3: Effects of treatment with paclitaxel or its vehicle on ICSS in male and female rats. Panels a-b show paclitaxel effects on total stimulations per component in males (a) and females (b). Horizontal axes: Time in days after initiation of treatment. Arrows indicate days for vehicle or paclitaxel administration. Vertical axes: ICSS performance expressed as the % pre-paclitaxel baseline number of reinforcements earned per 10-min component. All points show mean±SEM (for both panels, N=6 vehicle-treated and N=8 paclitaxel-treated rats), and filled points indicate significantly different from Day 0 pre-paclitaxel baseline. Panels c-d show full ICSS frequency-rate curves determined before (Pre-PTX) and 29 days after initiation of paclitaxel treatment. Horizontal axes: Brain stimulation frequency in Hz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). All points show mean±SEM (N=8 for both panels), and filled point in (d) indicates significantly different from Pre-PTX at that frequency. Statistical results are as follows: (a) No significant main effect of treatment [$F(1,12)=1.940$;

P=0.941], a significant main effect of time [F(24,288)=1.940; P=0.006], and no significant interaction [F(24,288)=0.514; P=0.098]. (b) No significant main effect of treatment [F(1,12)=4.508; P=0.055], a significant main effect of time [F(24,288)=2.128; P=0.002], and no significant interaction [F(24,288)=14.31; P=0.405]. (c) No significant main effect of treatment [F(1,6)=1.898; P=0.218], a significant main effect of frequency [F(9,54)=83.090; P<0.0001], and no significant interaction [F(9,54)=1.883; P=0.0744]. (d) No significant main effect of treatment [F(1,7)=1.660; P=0.239], a significant effect of frequency [F(9,63)=57.45; P<0.0001], and a significant interaction [F(9,63)=2.484; P=0.017].

number of stimulations per component over time in male and female rats. Paclitaxel did not significantly alter ICSS responding in either sex compared to vehicle-treated animals. Figure IV.3c and IV.3d show full ICSS frequency-rate curves before and 29 days after initiation of paclitaxel treatment in males and females, respectively. Paclitaxel did not significantly alter ICSS frequency-rate curves in males. In females, there was a significant treatment x frequency interaction (P=0.017); however, post hoc analysis indicated a significant but small decrease in ICSS responding at only one frequency (89 Hz). Treatment with paclitaxel vehicle did not significantly alter ICSS frequency-rate curves in males or females (data not shown).

Repeated morphine effects on ICSS responding in vehicle- vs. paclitaxel-treated male and female rats

Effects of repeated morphine treatment on the total-stimulations-per-component measure of ICSS during cumulative-dose testing are shown in Figures IV.4 and IV.5 for male and female rats treated initially with vehicle or paclitaxel, respectively. In general, repeated morphine treatment increased the ICSS-facilitating effects of morphine regardless of sex or vehicle/paclitaxel treatment. Thus, in the vehicle-treated males (Figure IV.4a), the initial exposure to morphine on Day 22 produced only a dose-dependent decrease in the total-stimulations-per-component measure of ICSS, but on Day 29, after six days of repeated 3.2 mg/kg/day morphine, ICSS was significantly increased by a dose of 3.2 mg/kg morphine. Additionally, ICSS rates after administration of 3.2 and 10 mg/kg morphine were significantly higher on Day 29 than on Day 22. Qualitatively similar effects were observed in vehicle-treated females (Figure IV.4b), and most importantly, 3.2 mg/kg morphine significantly facilitated ICSS in females on Day 29. Figure IV.4c-f highlights effects of 3.2 mg/kg morphine on full frequency-rate curves in vehicle-treated males and females on Day 22 (before repeated morphine) and Day

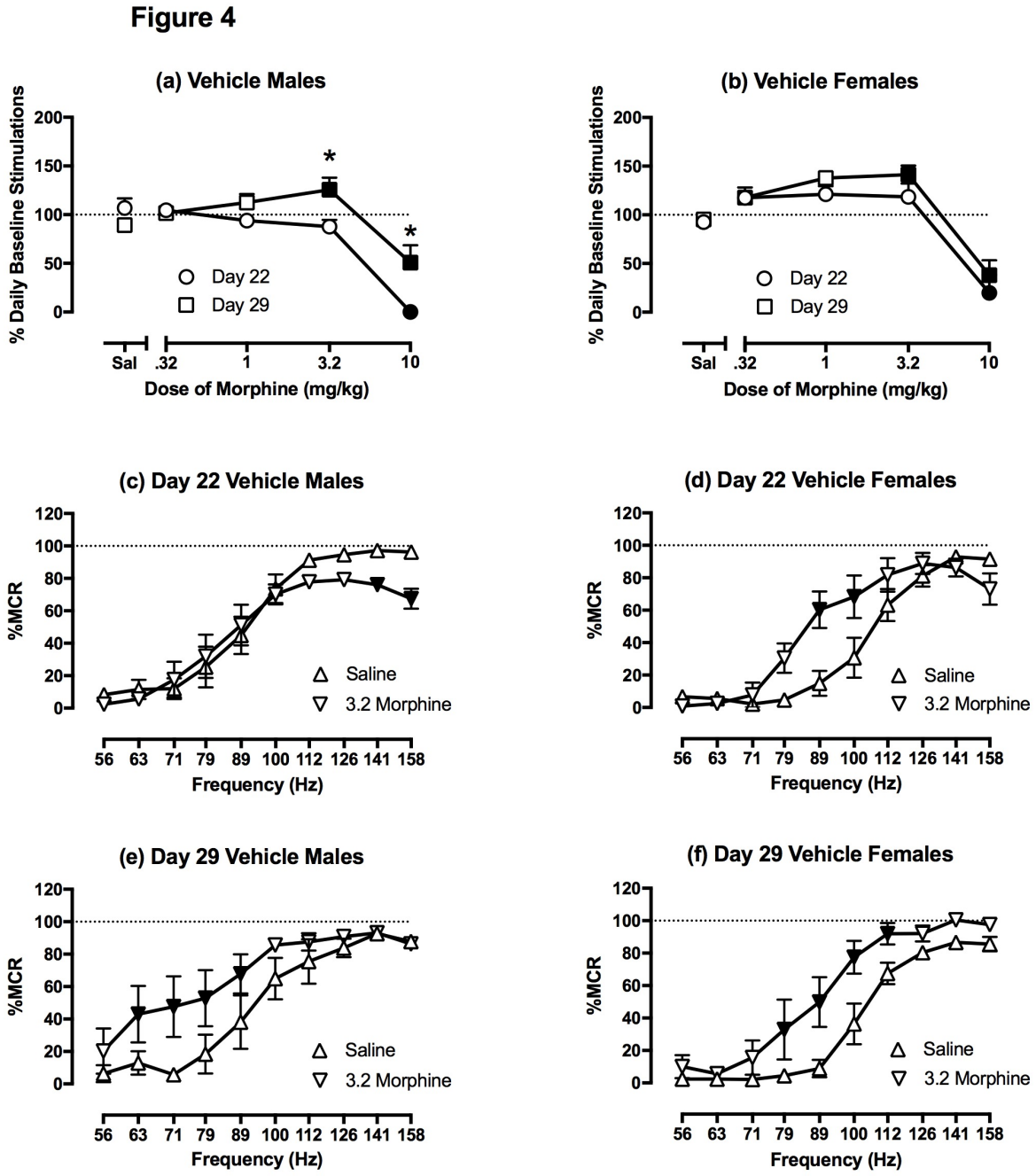

Figure IV.4: Effects of repeated morphine on ICSS in male and female rats treated with paclitaxel or vehicle. Top panels show the effects of cumulative morphine doses on total stimulations per component in males (a) and females (b). Horizontal axes: Cumulative doses of morphine in mg/kg. "Sal" = saline. Vertical axes: ICSS performance expressed the % daily-

baseline number of brain-stimulation reinforcements earned per 10-min component. Filled symbols indicate significantly different from "Sal" within a day, and asterisks indicate significant difference between days at a given morphine dose. Bottom panels show full ICSS frequency-rate curves determined after treatment with saline or cumulative 3.2 mg/kg morphine on Day 22 in males (c) and females (d) or on Day 29 in males (e) and females (f). Horizontal axes: Brain stimulation frequency in Hz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). Filled symbols indicate significant difference from "Saline" at a given frequency. All points show mean±SEM in 6 rats. Statistical results are as follows: (a) Significant main effects of treatment [$F(4,20)=38.45$; $P<0.0001$] and day [$F(1,5)=8.395$; $P=0.040$], and a significant interaction [$F(4,20)=7.344$; $P=0.001$]. (b) Significant main effect of treatment [$F(4,20)=28.160$; $P<0.0001$], no significant effect of day [$F(1,5)=3.280$; $P=0.130$], and no significant interaction [$F(4,20)=0.981$; $P=0.440$]. (c) No significant main effect of treatment [$F(1,5)=3.999$; $P=0.102$], a significant main effect of frequency [$F(9,45)=39.45$; $P<0.0001$], and a significant interaction [$F(9,45)=2.551$; $P=0.018$]. (d) No significant main effect of treatment [$F(1,5)=5.417$; $P=0.067$], but a significant main effect of frequency [$F(9,45)=51.850$; $P<0.0001$], and a significant interaction [$F(9,45)=9.864$; $P<0.001$]. (e) Significant main effects of treatment [$F(1,5)=9.001$; $P=0.030$] and frequency [$F(9,45)=24.74$; $P<0.0001$], but no significant interaction [$F(9,45)=2.028$; $P=0.058$]. (f) Significant main effects of treatment [$F(1,5)=10.40$; $P=0.023$] and frequency [$F(9,45)=73.280$; $P<0.0001$], and a significant interaction [$F(9,45)=3.254$; $P=0.004$].

29 (after repeated morphine). Relative to saline treatment on the respective test days, 3.2 mg/kg morphine only decreased high rates of ICSS maintained by high brain-stimulation frequencies on Day 22 in males (Figure IV.4c), but by Day 29, tolerance had developed to this rate-decreasing effect, and morphine increased ICSS rates across a broad range of frequencies (63-89 Hz; Figure IV.4e). In females, 3.2 mg/kg morphine significantly increased ICSS rates at two frequencies (89-100 Hz) on Day 22 (Figure IV.4c) and at four frequencies on Day 29 (Figure IV.4e).

In general, similar effects of repeated morphine were observed in paclitaxel-treated rats. Thus, in the paclitaxel-treated males (Figure IV.5a), the initial exposure to morphine on Day 22 produced only a dose-dependent decrease in the total-stimulations-per-component measure of ICSS, but on Day 29, after six days of repeated 3.2 mg/kg/day morphine, ICSS was significantly increased by a dose of 3.2 mg/kg morphine. Similarly, in females, morphine produced only a dose-dependent decrease in this measure of ICSS on Day 22, but cumulative doses of 1.0 and 3.2 mg/kg morphine significantly increased ICSS on Day 29. In both males and females, ICSS rates were significantly higher after 1.0 and/or 3.2 mg/kg morphine on Day 29 than on Day 22. Figure IV.5c-f highlights effects of 3.2 mg/kg morphine on full frequency-rate curves in paclitaxel-treated males and females on Day 22 (before repeated morphine) and Day 29 (after repeated morphine). Relative to effects of saline treatment, 3.2 mg/kg morphine only decreased high rates of ICSS maintained by high brain-stimulation frequencies on Day 22 in males (Figure IV.5c), but by Day 29, tolerance had developed to this rate-decreasing effects, and morphine increased ICSS rates at three frequencies (79-100 Hz; Figure IV.4e). In females, 3.2 mg/kg morphine significantly increased ICSS rates at three frequencies (71, 89, 100 Hz) on Day 22 but also decreased rates at the highest frequency of 158 Hz. On Day 29, 3.2 mg/kg morphine increased ICSS rates at 79-100 Hz with no evidence of rate-decreasing effects at any frequency.

Figure 5

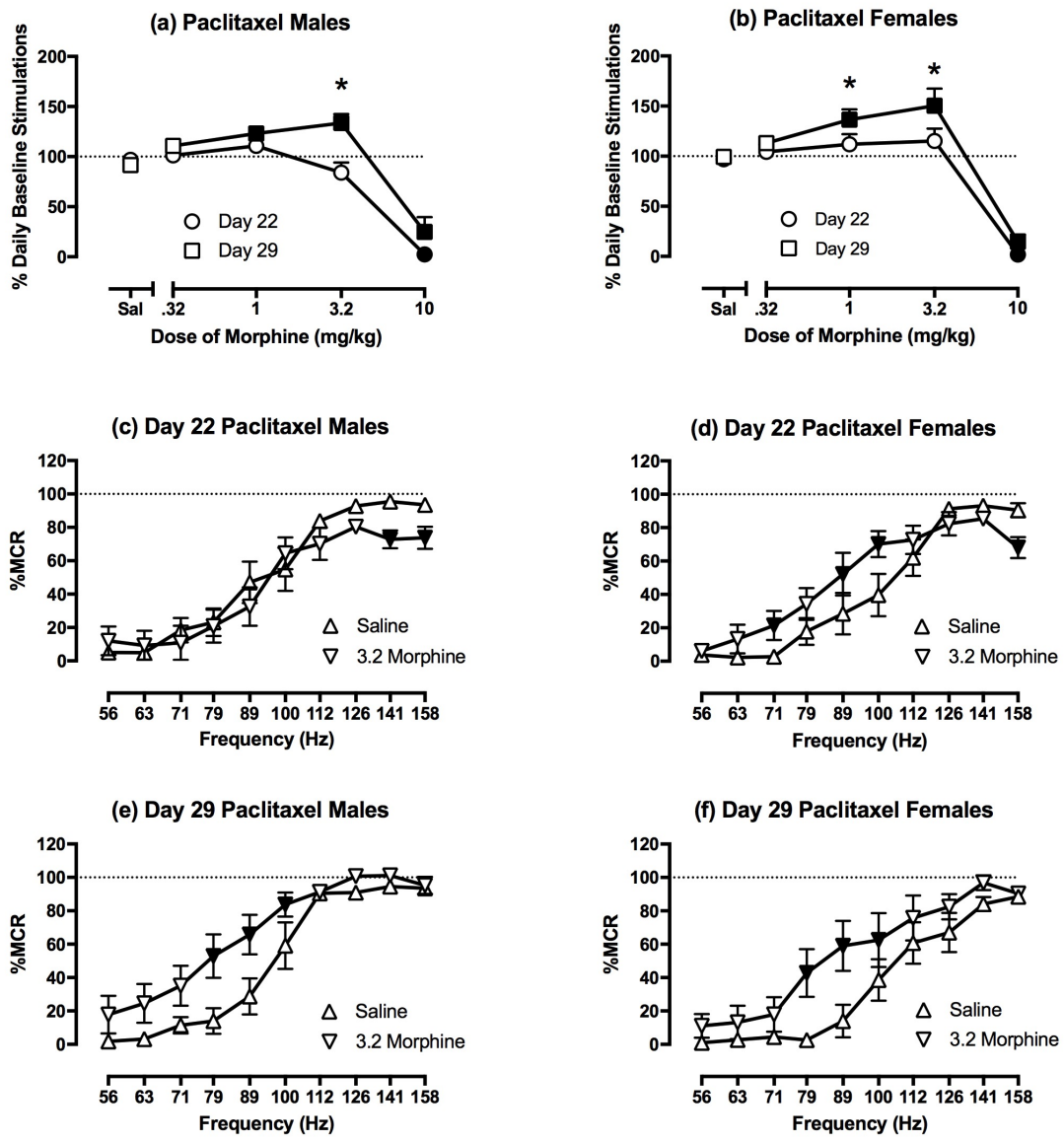

Figure IV.5: Effects of repeated morphine on ICSS in male and female rats treated with 2.0 mg/kg/day paclitaxel. Top panels show the effects of cumulative morphine doses on total stimulations per component in males (a) and females (b). Horizontal axes: Cumulative doses of morphine in mg/kg. "Sal" = saline. Vertical axes: ICSS performance expressed the % daily-baseline number of brain-stimulation reinforcements earned per 10-min component. Filled symbols indicate significantly different from "Sal" within a day, and asterisks indicate

significant difference between days at a given morphine dose. Bottom panels show full ICSS frequency-rate curves determined after treatment with saline or cumulative 3.2 mg/kg morphine on Day 22 in males (c) and females (d) or on Day 29 in males (e) and females (f). Horizontal axes: Brain stimulation frequency in Hz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). Filled symbols indicate significant difference from "Saline" at a given frequency. All points show mean±SEM in 8 rats. Statistical results are as follows: (a) Significant main effects of treatment [$F(4,28)=92.73$;$P<0.0001$] and day [$F(1,7)=6.884$; $P=0.035$], and a significant interaction [$F(4,28)=7.198$; $P=0.0004$]. (b) Significant main effects of treatment [$F(4,28)=59.980$; $P<0.0001$] and day [$F(1,7)=6.083$; $P=0.043$], and a significant interaction [$F(4,28)=3.16$; $P=0.029$]. (c) No significant main effect of treatment [$F(1,7)=1.632$; $P=0.242$], a significant effect of frequency [$F(9,63)=61.53$; $P<0.0001$], and a significant interaction [$F(9,63)=2.066$; $P=0.046$]. (d) No significant main effect of treatment [$F(1,7)=4.19$; $P=0.080$], but a significant effect of frequency [$F(9,63)=38.950$; $P<0.0001$], and a significant interaction [$F(9,63)=4.594$; $P=0.0001$]. (e) Significant main effects of treatment [$F(1,7)=15.85$; $P=0.005$] and frequency [$F(9,63)=47.48$; $P<0.0001$], and a significant interaction [$F(9,63)=2.281$; $P=0.028$]. (f) Significant main effects of treatment [$F(1,7)=10.22$; $P=0.015$], a significant effect of frequency [$F(9,63)=33.99$; $P<0.001$], and a significant interaction [$F(9,63)=2.662$; $P=0.011$].

In addition to these data from cumulative-dosing test sessions on Days 22 and 29, Figure IV.6 shows that data from the single-dose test sessions on Days 23-28 also indicated increasing levels of morphine-induced ICSS facilitation over time. Thus, repeated morphine produced a gradual increase in ICSS facilitation over days regardless of sex or saline/paclitaxel treatment. To investigate the role of sex as a determinant for morphine's effects on ICSS, vehicle- and paclitaxel-treated rats were combined since chemotherapy treatment did not alter morphine effects. Figure IV.7a shows the initial effects of morphine in opioid-naïve rats on Day 22. There was a sex difference in effects of 3.2 mg/kg morphine, which produced significant ICSS facilitation in females but not males. A higher dose of 10 mg/kg morphine decreased ICSS in both sexes. Figure IV.7b shows effects of morphine on Day 29 after the regimen of repeated morphine treatment. There was no longer a sex difference in opioid effects, and morphine-induced ICSS facilitation was enhanced in both males and females. Specifically, morphine produced significant ICSS facilitation at 1 and 3.2 mg/kg in both sexes while still producing ICSS depression at 10mg/kg.

Summary

Chapter IV compared the effects of repeated morphine treatment on ICSS and mechanical hypersensitivity in male and female rats treated with paclitaxel. There were three main findings. First, paclitaxel produced sustained mechanical hypersensitivity but little change in baseline ICSS performance in both males and females. Second, initial morphine treatment dose-dependently alleviated paclitaxel-induced mechanical hypersensitivity in both sexes, and repeated morphine produced modest but significant tolerance to this antinociceptive effect. Third, initial morphine treatment produced greater abuse-related ICSS facilitation in females than males, and repeated morphine treatment enhanced ICSS facilitation in both male and female

rats treated with either paclitaxel or its vehicle. Overall, these results suggest that this model of paclitaxel-induced neuropathy does not alter the trajectory of increasing opioid reward that occurs during initial exposure to repeated morphine. More generally, these results suggest that CINP is not protective against opioid reward, and repeated morphine treatment may simultaneously produce both tolerance to analgesic effects and increased vulnerability to iatrogenic opioid addiction.

Figure 6

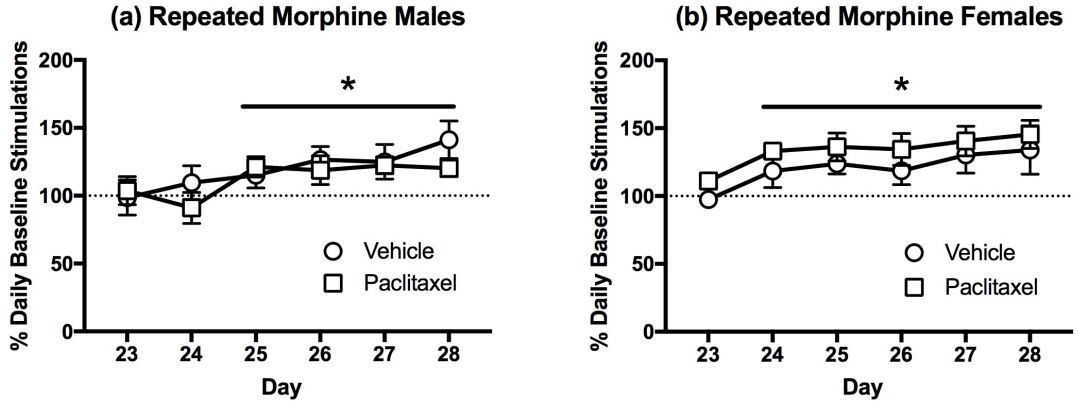

Figure IV.6: Effects of repeated 3.2 mg/kg/day morphine on ICSS performance in male and female vehicle- and paclitaxel-treated rats for single-dosing testing on Days 23-28. Horizontal axes: Time in days after initiation of paclitaxel treatment. Vertical axes: ICSS performance expressed the % baseline number of brain-stimulation reinforcements earned per 10-min component. All points show mean±SEM for N= 6 vehicle-treated rats and N=8 paclitaxel-treated rats in each panel. Line with a superior asterisk denotes Days statistically different from Day 23. Statistical results are as follows: (a) Significant main effect of day [$F(5,55)=7.551$; $P<0.0001$], but not of treatment [$F(1,11)=0.285$; $P=0.604$], and no significant interaction [$F(5,55)=1.596$; $P=0.177$]. (b) Significant main effect of day [$F(5,60)=4.704$; $P=0.001$], but not of treatment [$F(1,12)=1.525$; $P=0.241$], and no significant interaction [$F(5,60)=0.032$; $P=1.000$].

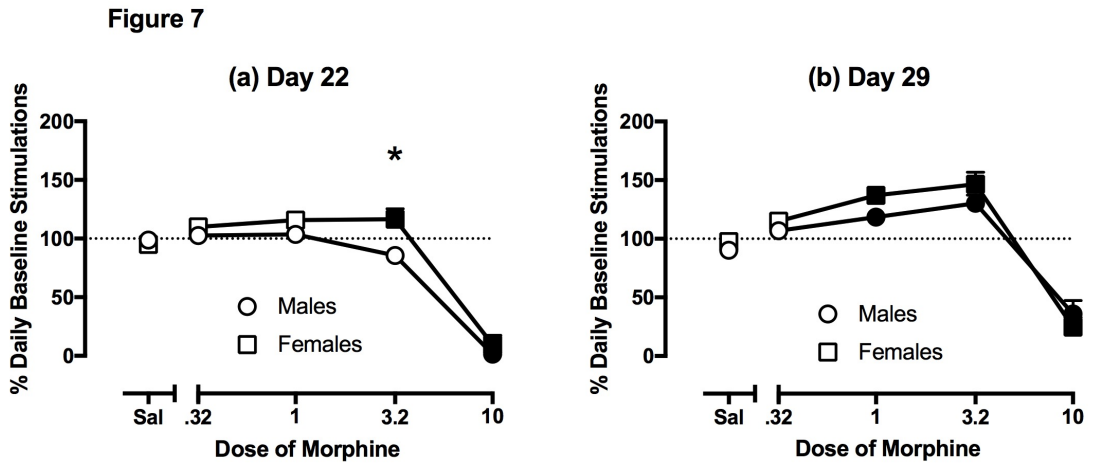

Figure IV.7: Effects of morphine on ICSS in male and female rats before and after repeated treatment with 3.2mg/kg/day morphine. Panels show the effects of cumulative morphine doses on total stimulations per component on Day 22 (a) and Day 29 (b). Horizontal axes: Cumulative doses of morphine in mg/kg. "Sal" = saline. Vertical axes: ICSS performance expressed as the % daily-baseline number of brain-stimulation reinforcements earned per 10-min component. All points show mean±SEM in 14 rats. Filled points denote significant difference from saline, asterisk denotes significant difference between males and females. Statistical results are as follows: (a) Significant main effects of dose [$F(4,104)=166.700$; $P<0.0001$] and sex [$F(1,26)=4.306$; $P=0.048$], and a significant interaction [$F(4,104)=3.408$; $P=0.012$]. (b) Significant main effect of dose [$F(4,104)=107.600$; $P<0.0001$] but not of sex [$F(1,26)=1.727$; $P=0.200$], and no significant interaction [$F(4,104)=2.041$; $P=0.094$].

Chapter V: Discussion

Summary

This dissertation evaluated the effect of chemotherapy treatment on motivated behaviors and opioid reward. The main findings of this evaluation are as follows: (1) Chemotherapy, at doses that produce robust and sustained mechanical hypersensitivity produce only weak or nonexistent depression of motivated behaviors. (2) There was no correlation between the expression of mechanical hypersensitivity and depression of motivated behaviors across individual animals, suggesting that these two effects of chemotherapy do not share common mechanisms of action. (3) Mechanical hypersensitivity, but not behavioral depression could be reversed with morphine. (4) The class of chemotherapeutic used in preclinical models is a determinant of the severity of effects on neuropathy-related endpoints and on the time course of these effects. (5) Chemotherapy does not protect against the rewarding effects of repeated morphine administration and does not alter the time course of the enhancement of reward with repeated morphine exposure. These findings suggest a need to revisit the mechanism outlined in the hypothesis and approach of this dissertation (Figure V.1). The revised hypothesis as a result of these studies and review of relevant literature are as follows: Administration of chemotherapy to rats induces mechanical hypersensitivity while failing to decrease mesolimbic dopamine signaling with consequent depressions in motivated behaviors and protection against morphine abuse-related effects. While apparent that chemotherapy can produce peripheral neuropathy, the data in this dissertation does not support the hypothesis that chemotherapy can produce chemotherapy-induced neuropathic pain (CINP) in rats.

Figure V.1

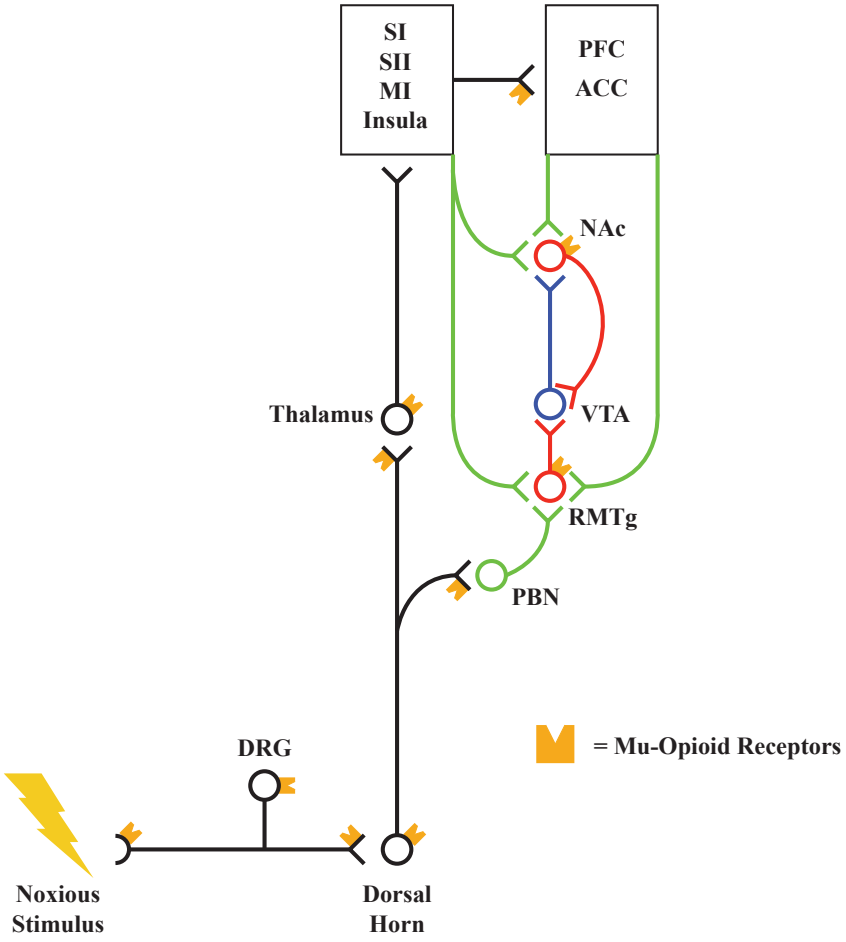

Figure V.1: Data obtained in dissertation studies modifies the hypothesis of chemotherapy-induced neuropathy effects on mesolimbic dopamine signaling. The failure of chemotherapies to depress intracranial self-stimulation (ICSS) targeting mesolimbic dopaminergic neurons, to depress food-maintained responding, and to attenuate the rewarding effects of repeated morphine administration does not support the hypothesis that neuropathy-inducing chemotherapeutics are a sufficient noxious stimulus to depress ventral tegmental area (VTA) to nucleus accumbens (NAc) mesolimbic dopamine signaling by any mechanism proposed. Repeated morphine exposure produced enhanced rewarding effects regardless of the presence or absence of

chemotherapy-induced neuropathy. A possible explanation for observing increasing morphine reward could be due to sensitization of mu-opioid receptor (MOR) positive neurons within the rostromedial tegmentum (RMTg) or NAc and/or desensitization of MOR positive neurons within brain regions responsible for the negative effects of MOR agonists such as sedation and motor impairment. SI, primary somatosensory cortex; SII, secondary somatosensory cortex; MI, primary motor cortex; ACC, anterior cingulate cortex; PFC, prefrontal cortex; PBN, parabrachial nucleus.

Implications of Chapter II:

Chapter II compared effects of paclitaxel treatment in rats on (a) mechanical sensitivity of paw-withdrawal responses, and (b) positively reinforced operant responding maintained by electrical brain stimulation in an intracranial self-stimulation (ICSS) assay or by food. There were three main findings. First, paclitaxel doses sufficient to produce mechanical hypersensitivity did not reliably depress ICSS in male or female rats. Second, analysis of data from individual rats indicated that the degree of behavioral suppression in ICSS did not correlate with mechanical sensitivity. The lack of correlation between mechanical sensitivity and behavioral suppression suggests that mechanical hypersensitivity does not cause behavioral suppression, may have different underlying mechanisms than behavioral suppression, and may not serve as a useful surrogate measure for clinically relevant signs of behavioral depression in CINP. Third, food-maintained responding was more sensitive to detect chemotherapy-induced depression of motivated behavior than ICSS.

Effects of paclitaxel on body weight, mechanical sensitivity, and intra-epidermal nerve fiber density.

The effects of paclitaxel reported here agree with previous studies in rodents that examined the time course and extent of mechanical hypersensitivity following paclitaxel treatment (Polomano et al., 2001; Pascual et al., 2010; Hwang et al., 2012; Boyette-Davis et al., 2011; Ko et al., 2014; Toma et al., 2017). For example, Polomano et al., 2001, found that four injections of 2.0 mg/kg/day paclitaxel on alternating days produced significant mechanical hypersensitivity for four weeks. With regard to sex differences in paclitaxel effects, one previous study found that female mice were more sensitive than males to paclitaxel-induced cold hypersensitivity (Naji-Esfahani et al., 2016); however, as in previous studies, paclitaxel-induced

mechanical hypersensitivity was similar in males and females (Hwang et al., 2012; Naji-Esfahani et al., 2016) and correlated with decreases in intra-epidermal nerve fiber densitiy (Boyette-Davis et al., 2011a; Ko et al., 2014). The present study extends on these results by showing that a 0.5 log unit higher paclitaxel dose (four injections of 6.0 mg/kg/day) produced sufficient weight loss to require euthanasia in most rats in accordance with moribundity criteria in the animal use protocol.

Effects of paclitaxel on intracranial self-stimulation (ICSS)

Behavioral depression is a cardinal sign of clinically relevant pain (Dworkin et al., 2008) and the importance of pain-depressed behaviors in guiding diagnosis and treatment of human pain is growing given concerns about reliance on verbal pain reports (Sullivan and Ballantyne, 2016). ICSS is one type of behavioral baseline that can be used to evaluate preclinical expression and treatment of pain-related behavioral depression and functional impairment in rats (Pereira Do Carmo et al., 2009; Negus et al., 2010; Negus, 2013), and the principal goal of this study was to test the working hypothesis that paclitaxel treatment regimens sufficient to produce mechanical hypersensitivity would also depress ICSS. Our results do not support this hypothesis.

The weak effects of paclitaxel on ICSS cannot be attributed to a general lack of ICSS sensitivity to putative pain states. Consistent with the expression of both depressed behavior and depressed mood in many human pain states, ICSS in rats can be depressed transiently (hours to days) by inflammatory noxious stimuli that include intraperitoneal injection of dilute acid, paw incision, and intraplantar administration of complete Freund's adjuvant (Pereira Do Carmo et al., 2009; Ewan and Martin, 2014; Leitl et al., 2014b; Brust et al., 2016). Moreover, these examples of pain-related depression of ICSS can be reversed by clinically effective analgesics (e.g. opioids

and nonsteroidal anti-inflammatory drugs) but not by drugs (e.g. centrally acting kappa opioid receptor agonists) that produce motor impairment and appear as false positives in conventional preclinical assays (Negus, 2013). However, the effectiveness of neuropathic manipulations to decrease ICSS has been less consistent. For example, intraplantar formalin administration produced a sustained and analgesic-reversible depression of ICSS for up to two weeks (Leitl and Negus, 2016), but this finding was not replicated in a later study in our laboratory (Lazenka and Negus, unpublished observations), and spinal nerve ligation as a surgical neuropathy model failed to alter ICSS (Ewan and Martin, 2014; Ewan and Martin, 2017). Consistent with the effects of spinal nerve ligation, paclitaxel treatments sufficient to produce mechanical sensitivity in the present study failed to produce significant or reliable decreases in ICSS in either male or female rats. It remains possible that other paclitaxel treatment regimens might be effective to decrease ICSS; however, given the severe weight loss produced by repeated 6.0 mg/kg/day paclitaxel in this study, there is a narrow window for more intensive treatments, and pilot studies conducted by us (e.g. a second round of repeated 4x2.0 mg/kg/day paclitaxel) were also not effective (data not shown).

One interpretation of these results is that ICSS in rats is not useful for assessment of any neuropathic pain produced by these paclitaxel treatment regimens. Electrical brain stimulation can function as an extremely efficacious reinforcer (Negus and Miller, 2014), and it is possible that other behaviors (e.g. operant responding for food) may be more susceptible than ICSS to depression by paclitaxel. This possibility was addressed experimentally as described below, and consistent with this possibility, 4 injections of 2.0 mg/kg/day produced significant if transient decreases in body weight for males in the present study. However, ICSS was used here for two reasons in addition to its previously demonstrated utility in other studies of pain-depressed

behavior. First, different frequencies of electrical brain stimulation can be used to efficiently maintain a range of low-to-high rates of responding that are stable over time and sensitive to perturbation by a variety of treatments (Negus and Miller, 2014). Second, ICSS relies on direct stimulation of neural circuits that underlie reinforcement independent of common sensory modalities (e.g. taste), and as a result, the procedure has also been used to examine effects of experimental manipulations on reward system function and neurobiology of motivation (Carlezon and Chartoff, 2007). Perhaps the most common use of ICSS in this regard has been to evaluate abuse liability of drugs, and drugs of abuse typically increase (or "facilitate") responding suggestive of enhanced reward system function; however, ICSS has also been used to examine effects of manipulations that impair reward system function and contribute to affective signs of anhedonia and depression (Carlezon and Chartoff, 2007; Negus and Miller, 2014). Notably, paclitaxel failed to reduce even low rates of ICSS maintained by low brain-stimulation frequencies that function as weak reinforcers. As such, these results provide no evidence for an effect of paclitaxel treatment on reward system function.

An alternative and more controversial interpretation of these findings is that conventional paclitaxel treatment regimens produce little or no pain in rodents. These treatment regimens were initially validated behaviorally by their effectiveness to produce hypersensitive withdrawal responses from thermal and mechanical stimuli (e.g. Polomano et al., 2001), but hypersensitive withdrawal responses are not a common sign of human chronic pain in general or chemotherapy-induced neuropathic pain in particular (Dworkin et al., 2008; Golan-Vered and Pud 2013). Moreover, although thermal and/or mechanical allodynia is observed in a subset of human neuropathy patients, it is measured not as hypersensitivity of withdrawal responses but as hypersensitivity of verbal endorsement of subjective pain. Use of the common term "allodynia"

to describe these different behaviors is problematic for translational research as preclinical and clinical studies are measuring very different endpoints, despite using the same stimulus. More generally, it may be inappropriate to interpret hypersensitive withdrawal responses as evidence of pain, and the present results suggest that even if paclitaxel-induced hypersensitivity of withdrawal responses is associated with a pain experience in rodents, that experience is not of a sufficient type or intensity to depress ICSS.

Effects of paclitaxel on food-maintained operant responding

Like ICSS, food-maintained operant responding can also serve as a stable behavioral baseline for preclinical studies of pain-depressed behavior, and previous studies have shown depression of food-maintained responding by noxious inflammatory stimuli that include intraperitoneal injection of dilute acid, intraplantar complete Freund's adjuvant, paw incision, and laparotomy (Martin et al., 2004; Martin et al., 2005; Ewan and Martin, 2014; Warner et al., 2015; Okun et al., 2016). As with depression of ICSS, depression of food-maintained responding is generally more transient than mechanical hypersensitivity, and it can be blocked or reversed by opioid analgesics and non-steroidal anti-inflammatory drugs. In contrast, food-maintained responding was not reduced by spinal nerve ligation as a surgical neuropathy manipulation and only transiently decreased by intraplantar formalin administration (Ewan and Martin, 2014; Freitas et al., 2015; Okun et al., 2016). In the present study, paclitaxel produced a significant though modest depression of food-maintained responding by the end of the 29-day treatment period, and as such, paclitaxel treatment was both (a) more effective than other neuropathy manipulations to decrease food-maintained responding, and (b) more effective to decrease food-maintained responding than ICSS. These findings suggest that food-maintained responding may be a useful behavioral baseline for studies of paclitaxel-induced and pain-related behavioral

depression; however, paclitaxel-induced decreases in food-maintained responding may result from paclitaxel effects other than pain, such as disrupted taste and decreased palatability of food reward (Strasser et al., 2008; Cohen et al., 2016). Future studies will be required to investigate this possibility. Moreover, as with ICSS, the lack of correlation between mechanical sensitivity and depression of food-maintained responding suggests that mechanical sensitivity may not be a useful surrogate measure for clinically relevant signs of behavioral depression and functional impairment in neuropathic pain.

Implications for Chapter III:

Chapter III compared the effects of the four neuropathic classes of chemotherapeutics using paclitaxel, vincristine, oxaliplatin, and bortezomib on mechanical hypersensitivity and motivated behavior. There were three main findings. First, differential effects were observed with the four different chemotherapeutics on the behavioral endpoints of mechanical hypersensitivity and food-maintained responding. All four drugs produced dose-dependent and sustained mechanical hypersensitivity that emerged during initial treatment and lasted until the end of study, three weeks after termination of treatment. However, the magnitude of mechanical sensitivity varied across drugs with paclitaxel and oxaliplatin producing robust hypersensitivity, vincristine producing moderate hypersensitivity, and bortezomib producing mild hypersensitivity. The time course and magnitude of decreases in food-maintained responding also varied across drugs. Paclitaxel produced weak but significant decreases at later time points in food-maintained responding. Vincristine produced severe decreases at early time points in food-maintained responding, but behavior recovered one week after the final injection. Oxaliplatin produced weak decreases at early time points in food-maintained responding that

recovered shortly after the final injection. Bortezomib produced limited decreases in food-maintained responding only during the injection period. Second, depression of behavior was not observed at the end of the study (Days 31-41 after initiation of chemotherapy treatment) in evaluation of food-reward efficacy using a behavioral economic approach to determine demand curves. This buttresses the notion in Chapter II that chemotherapy produces weak or non-existent long-term behavioral depression. Only paclitaxel administration was sufficient to produce both mechanical hypersensitivity and decreases in food-maintained responding at later time-points. With regard to producing both pain-stimulated and pain-depressed effects, paclitaxel appears to be the most promising model of CINP compared to vincristine, oxaliplatin, and bortezomib, although even with paclitaxel, behavioral depression was small and not apparent in the behavioral economic analysis. Third, morphine was sufficient to reverse paclitaxel-, vincristine-, oxaliplatin-, and bortezomib-induced hypersensitivity but was unable to reverse paclitaxel-induced behavioral depression. This result further divides the possible mechanisms underlying hypersensitivity and behavioral depression.

Effects of paclitaxel, vincristine, oxaliplatin, and bortezomib on body weight and mechanical sensitivity in free-feeding rats

There have been two previous studies that have compared the effects of different classes of chemotherapeutics on preclinical nociceptive endpoints (Janes et al., 2013; Boehmerle et al., 2014). The present study addressed one limitation in the previous comparisons by utilizing the same treatment regimen for all four drugs and in testing several doses for each drug including a maximum tolerated dose. This enabled comparisons of severity and time course for chemical neuropathic effects. The effects of 2.0 mg/kg paclitaxel on body weight and mechanical sensitivity thresholds reported here agree with previous studies in rodents that examined the time

course and extent of mechanical hypersensitivity following paclitaxel treatment (Polomano et al., 2001; Pascual et al., 2010; Boyette-Davis et al., 2011a; Hwang et al., 2012; Ko et al., 2014; Toma et al., 2017; Legakis et al., 2018). Vincristine produced sustained mechanical hypersensivity and decreases in body weight with the high doses of 0.125 and 0.25 mg/kg, which agrees with previous reports (Ji et al., 2013; Linglu et al., 2014; Amoateng et al., 2015). Oxaliplatin produced sustained mechanical hypersensivity and decreases in body weight with the high doses of 2.5 and 5.0 mg/kg, which agrees with previous reports (Kawashiri et al., 2011; Liu et al., 2013; Fujita et al., 2015). Bortezomib produced weak but significant mechanical hypersensitivity with no change in body weight, which agrees with previous reports (Chiorazzi et al., 2013; Janes et al., 2013; Yamamoto et al., 2015). While comparisons to previous studies in the field are somewhat difficult due to the differing doses and treatment regimens, all four chemotherapies produced mechanical hypersensitivity at high doses, with paclitaxel and oxaliplatin producing robust hypersensitivity and vincristine and bortezomib producing weaker mechanical hypersensitivity. Additionally, chemotherapy treatments had differential effects on body weight with vincristine and oxaliplatin producing sustained decreases in body weight and paclitaxel and bortezomib producing no significant decreases in body weight. Paclitaxel and bortezomib at doses higher than the maximal tolerated doses produce severe decreases in body weight (Legakis et al., 2018). The ranking of chemotherapies in terms of ability to produce hypersensitivity from most severe to least severe at the final time point was as follows: oxaliplatin, paclitaxel, vincristine, bortezomib.

Effects of morphine on mechanical allodynia

Allodynia in this experiment was defined as significant mechanical hypersensitivity in chemotherapy-treated rats compared to vehicle-treated rats. The following doses produced

mechanical allodynia at the final time point: 2.0 mg/kg paclitaxel, 0.125 and 0.25 mg/kg vincristine, 2.5 and 5.0 mg/kg oxaliplatin, and 0.0625 and 0.25 mg/kg bortezomib. For all doses and different chemotherapies tested, 3.2 and 10.0 mg/kg morphine were sufficient to reverse mechanical allodynia while 1.0 mg/kg was not. The severity of mechanical allodynia did not change morphine's potency to produce antinociception as has been reported for other pain states such as warm-water tail withdrawal at differing thermal intensities (Maguire and France, 2014; Cornelissen et al., 2018). Standardizing the effects of chemotherapy by comparing morphine doses as %MPE did not shift the ED50s for morphine's antinociceptive effects. These results with morphine on chemotherapy-induced hypersensitivity agree with previous studies conducted in rodents with paclitaxel (Pascual et al., 2010; Hwang et al., 2012; Neelakantan et al., 2016), vincristine (Lynch et al., 2004; Saika et al., 2009; Park et al., 2010), and oxaliplatin (Kanbara et al., 2014; Michot et al., 2014; Kim et al., 2016). No previous studies have investigated the antinociceptive effects of morphine on bortezomib-induced mechanical hypersensitivity.

Chemotherapy effects on food-maintained responding

Behavioral depression is a cardinal sign of clinically relevant pain (Dworkin et al., 2008) and the importance of pain-depressed behaviors in guiding diagnosis and treatment of human pain is growing given concerns about reliance of verbal pain reports (Sullivan and Ballantyne, 2016). Positively reinforced operant behavior provides one type of baseline behavior to evaluate preclinical expression and treatment of pain-related behavioral depression and functional impairment (Pereira Do Carmo et al., 2009; Negus, 2013).

In operant assays with food pellets serving as positive reinforcement, divergent effects among the class of chemotherapeutics were observed. Four injections of 2.0 mg/kg paclitaxel produced weak but chronic depressions in food-maintained responding. Although this

depression of responding was not reflected in depression of food reinforcing efficacy following neuropathy as determined through a behavioral-economic analysis, this depression persisted past six weeks since the initiation of treatment. These results agree with previous results from our laboratory (Chapter II, unpublished) and results from a previous study that tested a cumulative dose of 2.0 mg/kg paclitaxel in mice (Mustafa et al., 2013). In Mustafa et al., 2013, mice were trained to nose poke for food reinforcement through a ring that can be heated to high temperatures. In mice treated with paclitaxel, there were no decreases in operant behavior for food reinforcement, however there were decreases when the thermal ring was calibrated to 37°C (acting as a punisher) compared to vehicle, suggesting paclitaxel-induced hypersensitivity to thermal stimuli as opposed paclitaxel-induced depressions of behavior. Maximum tolerated doses of vincristine, oxaliplatin, and bortezomib sufficient to produce mechanical allodynia did not significantly depress food-maintained responding past two weeks from the initiation of chemotherapy. Oxaliplatin and bortezomib produced acute and transient decreases in operant behavior that resolved with cessation of drug delivery while vincristine-induced behavioral depression resolved one week after cessation of treatment. No previous studies have investigated the effects of bortezomib or vincristine in operant behavioral assays. Two studies from the same lab have investigated oxaliplatin in an operant assay designed to test hypersensitivity to mechanical stimuli (Ling et al., 2017) or cold stimuli (Abd-Elsayed et al., 2015) while emitting nose pokes for condensed milk (reinforcer). In both procedures, a cumulative dose of 10 mg/kg of oxaliplatin (20mg/kg was tested in this study) in rats produced behavioral depression in the presence of punishing stimuli for one month (mechanical) or two months (cold) after cessation of oxaliplatin treatment. Effects of oxaliplatin on condensed milk-maintained responding were not tested in the absence of mechanical or cold punishing stimuli. These results suggest oxaliplatin-

induced hypersensitivity as opposed to oxaliplatin-induced depressions of behavior. Overall, these results provide weak evidence for sustained behavioral depression after paclitaxel treatment and no evidence of sustained behavioral depression after vincristine, oxaliplatin, or bortezomib treatment.

Chemotherapy effects on sucrose preference

The chemotherapy-induced decreases in food-maintained responding observed may be a result of chemotherapy's effects other than pain, such as disrupted taste and decreased palatability of food reward (Strasser et al., 2008; Cohen et al., 2016). In addition, chemotherapy has been shown to increase symptoms and diagnoses of clinical depression (Pedersen et al., 2007; Hjorth et al., 2012; Lynn et al., 2017). Decreases in sucrose preference in rodents have been linked to other preclinical endpoints modeling depression and can be reversed with clinically effective antidepressants (Sofia and Knobloch, 1976; Brenes Saenz et al., 2006). Paclitaxel, vincristine, oxaliplatin, and bortezomib all failed to alter 2% sucrose preference four weeks after the initiation of treatment, suggesting depression-like effects of chemotherapy are not present at this time and cannot account for the decreases in food-maintained responding in the paclitaxel-treated rats at this time. One previous study investigated the effects of paclitaxel on 2% sucrose preference (Toma et al., 2017). In Toma et al., 2017, sucrose preference was decreased in mice treated with a cumulative dose of 32 mg/kg paclitaxel during and shortly following the injection cycle compared to vehicle-treated mice. These decreases were abolished by one week following completion of the treatment regimen which would agree with the results presented here observing no difference in 2% sucrose preference three weeks after the completion of 8 mg/kg paclitaxel. No previous studies have investigated the effects of vincristine, oxaliplatin, or bortezomib on sucrose preference. While it is difficult to rule out the

potential sensory impact of chemotherapy on taste, chemotherapy-treated rats displayed a strong preference for sucrose to a similar degree as vehicle-treated rats. Future studies testing a range of sucrose concentrations could better elucidate chemotherapy effects on taste.

Morphine and Nortriptyline effects on paclitaxel- and vincristine-depressed operant responding

Despite being relatively ineffective analgesics for neuropathic pain, opioid analgesics are often used in part to treat CINP in cancer survivors. In the IASP review and recommendation of treatments for neuropathic pain, opioid analgesics are determined to have limited effectiveness and are considered second-line treatment options (Dworkin et al 2010). There have been randomized clinical trials that have demonstrated, at best, equivocal pain reduction in neuropathic pain patients compared to tricyclic antidepressants (TCAs) such as nortriptyline and serotonin-norepinephrine reuptake inhibitors (SNRIs) (Arner and Meyerson, 1988; Raja et al., 2002; Eisenberg et al., 2005). Recommendations aside, opioid analgesics are often being prescribed for CINP in cancer survivors, possibly for reasons related to continuation of treatments with recurrent cancer or chemotherapy-related pain as at least 70% of those suffering from invasive cancer are prescribed chronic opioid analgesics for pain (Plante and VanItallie, 2010). Another possible reason is that with no fully effective analgesics for CINP, following the recommendations of the World Health Organization's analgesic step ladder would encourage the addition of opioid analgesics in persisting pain (World Health Organization, 1986). As a further analgesic issue, CINP does not appear to prevent or attenuate the prevalence of opioid analgesic tolerance or opioid-induced hyperalgesia in patients (Devulder, 1997; Ossipov et al., 2003; Chang et al., 2007).

In Chapter III studies, neither acute morphine (1.0 or 3.2 mg/kg) nor repeated nortriptyline (3.2 mg/kg) was sufficient to restore paclitaxel-induced behavioral depression. The doses of morphine chosen for this phase of studies were determined in Chapter III experiments where 1.0 mg/kg morphine did not reverse chemotherapy-induced allodynia but 3.2 mg/kg fully reversed chemotherapy-induced allodynia. 3.2 mg/kg nortriptyline was chosen based on previous studies and ongoing unpublished studies in which acute nortriptyline failed to reverse depression of ICSS by an acute i.p. acid noxious stimulus, but repeated nortriptyline did alleviate acid-induced ICSS depression (Rosenberg et al., 2013; Legakis and Negus, unpublished results). While neither drug was able to restore depressed operant responding to rates of vehicle, both doses of morphine and 3.2 mg/kg nortriptyline eliminated the difference between the groups by having larger rate-decreasing effects in vehicle-treated rats. It is difficult to say if this finding could be signature of a moderately effective analgesic for CINP or if the behavioral output observed is the maximum possible output under these drug conditions.

Chemotherapy effects on balance beam performance

An often-overlooked component of clinical chemotherapy-induced peripheral neuropathy is disrupted proprioception. Disruptions to proprioception have been observed in patients experiencing chemotherapy-induced neuropathy as evidenced by increased falls (Tofthagen et al., 2012a; Gewandter et al., 2013) and balance impairment (Sarosy et al., 1992; Wampler et al., 2007; Hile et al., 2010; Tofthagen et al., 2012b; Kneis et al., 2016). This sign of neuropathy can be crucial to patients' quality of life as falls are one of the most predictive causes of morbidity and mortality (Johnson et al., 2014). In preclinical rodent studies, both paclitaxel (Peters et al., 2007; Callizot et al., 2008) and vincristine (Callizot et al., 2008) can produce motor and proprioception deficits on the rotarod test. The hypothesis that neuropathic chemotherapy

treatment can disrupt proprioception and motor output is also supported by evidence of large fiber and motor neuropathy as well as sensory neuropathy in rodents (Cliffer et al., 1998; Peters et al., 2007).

Chapter III discusses the first studies to test the effects of paclitaxel, vincristine, or bortezomib on proprioception and/or motor output using balance beam performance. Balance beam performance has been utilized in rodent studies to look at other forms of neurological deficits (Metz et al., 2000; Luong et al., 2011) as well as one study investigating the effects of oxaliplatin (Taleb et al., 2017). The work presented in this dissertation utilized an experimental design and endpoints both similar to and wholly distinct from previous studies. For example, in Taleb et al., 2017, weak disruptions in proprioception were observed two weeks following the final administration of repeated 4 mg/kg oxaliplatin (32 mg/kg cumulative dose) using the endpoint of "mean grade of first paw misplacement" on a scale of 0 – 20, a relatively subjective qualitative scoring system (Taleb et al., 2017). This study implemented a quantitative endpoint that could assess the effects of the four chemotherapies.

Paclitaxel, vincristine, and oxaliplatin all produced disrupted balance beam performance in terms of significantly increasing the time to cross beams for at least one time point of the study compared to vehicle treatment. Paclitaxel produced increased cross times for the medium beam on Days 15 and 22. Vincristine increased cross times on the large and medium beams on Day 8, and oxaliplatin increased cross times only on the large beam on Day 15. Bortezomib failed to alter cross time at any time on any beam. For balance beam performance results with paclitaxel and vincristine, the results presented here support data from rotarod studies that suggest that these drugs can decrease proprioception and/or motor output (Peters et al., 2007; Callizot et al., 2008). The results presented here for effects of oxaliplatin on balance beam

performance mostly support previous data using a qualitative endpoint, (Taleb et al., 2017) with oxaliplatin producing disruptions to performance 1-2 weeks following completion of drug administration, with disagreements in the duration of these effects. In Taleb et al., 2017, these effects were sustained and lasted in the order of months. In this study, disruptions were only observed at the Day 15 timepoint. The potential explanations for this discrepancy are largely based on differences in experiental designs as the endpoints and drug administration regimens were quite different between the two experimental parameters. No study has investigated the effects of bortezomib on proprioception and/or motor output in balance beam or rotarod performance studies. The results presented here do not provide any evidence of disruptions in balance beam performance with four injections of 0.25 mg/kg bortezomib.

The disruptions in balance beam performance produced by paclitaxel, vincrsitine, and oxaliplatin can be considered weak compared to spinal cord lesions, cortical lesions, and a murine model for Huntington's Disease that produce more dramatic changes in common endpoints of balance beam performance (Fox et al., 1998; Carter et al., 1999; Metz et al., 2000). The results for the effects of each chemotherapy on balance beam performance don't fully resemble the severity and time course of effects on mechanical sensitivity or on operant responding and suggest that chemotherapy-related effects may have different mechanisms for all three endpoints.

The disruptions in balance beam performance can be explained by neuropathy of non-nociceptive primary afferents carrying proprioception information and/or by neuropathy of motor efferents carry motor output information. There is evidence for neuropathy of both large fiber primary afferents (Cliffer et al., 1998; Peters et al., 2007) and of motor efferents (Authier et al., 2000). Additionally, one reason why the effects of chemotherapy may be more sustained in

assays of mechanical sensitivity (thought to be governed primarily by neuropathy of small fiber nociceptors afferents) is due to differences in fiber type susceptibility and response neuropathic stimuli (Menorca et al., 2013).

Implications for Chapter IV:

Chapter IV compared the effects of repeated morphine treatment on ICSS and mechanical hypersensitivity in male and female rats treated with paclitaxel. There were three main findings. First, paclitaxel produced sustained mechanical hypersensitivity but little change in baseline ICSS performance in both males and females. Second, initial morphine treatment dose-dependently alleviated paclitaxel-induced mechanical hypersensitivity in both sexes, and repeated morphine produced modest but significant tolerance to this antinociceptive effect. Third, initial morphine treatment produced greater abuse-related ICSS facilitation in females than males, and repeated morphine treatment enhanced ICSS facilitation in both male and female rats treated with either paclitaxel or its vehicle and eliminated the sex difference. Overall, these results suggest that this model of paclitaxel-induced neuropathy does not alter the trajectory of increasing opioid reward that occurs during initial exposure to repeated morphine. More generally, these results suggest that CINP is not protective against opioid reward, and repeated morphine treatment may simultaneously produce both tolerance to analgesic effects and contribute to iatrogenic opioid addiction.

Effects of paclitaxel on mechanical sensitivity and ICSS

The effects of paclitaxel on mechanical sensitivity thresholds and ICSS agree with the findings reported here in Chapter II.

Effects of morphine on paclitaxel-induced mechanical hypersensitivity

Although sex differences in morphine antinociception have been reported in other preclinical injury models in rats (Boyer et al., 1998; Cicero et al., 2002; Craft, 2008), the present results agree with a previous report that failed to observe a sex difference in morphine potency to alleviate paclitaxel-induced mechanical hypersensitivity (Hwang et al., 2012). The present results extend these findings in two ways. First, to our knowledge, this is the the first study to report tolerance to morphine anti-allodynia in paclitaxel-treated rats receiving repeated morphine treatement; however, these findings agree with previous reports of tolerance to morphine anti-allodynia in rats studied using other nerve-injury models (Bulka et al., 2002; Ledeboer et al., 2006). It is also important to note that, although anti-allodynia in preclinical studies is often interpreted as evidence for potential analgesic effects in humans, there are several examples of poor translation between preclinical anti-allodynia and clinical analgesia for treatment of CINP (Xiao et al., 2009; Tatsushima et al., 2011; Paton et al., 2017). Thus, tolerance to morphine anti-allodynia shown here is only suggestive of a potential for tolerance to morphine analgesia in human patients with chemotherapy-induced neuropathic pain.

Second, the similarity in morphine potency on Days 22 and 29 in rats treated with repeated saline agrees with the similar baseline levels of hypersensitivity on those two days to suggest no progression in the underlying neuropathic pain state between these two times. These findings also suggest that the decrease in morphine potency after repeated morphine treatment reflected tolerance and not neuropathy progression.

Effects of morphine on ICSS

ICSS procedures have a record of predictive validity similar to that of drug self-administration procedures for preclinical abuse-liability assessment, and most drugs that

facilitate ICSS also have abuse liability in humans (Wise, 1996; Negus, 2013; Negus and Miller, 2014). We reported previously that repeated morphine treatment produces a progressive increase in expression of morphine reward in the ICSS procedure, and this trajectory of increasing opioid reward is not altered by a repeated acute-pain stimulus administered in conjunction with morphine (Miller et al., 2015). This preclinical finding agrees with clinical evidence that mu opioid receptor agonists produced more robust aversive effects and weaker euphoric effects in opioid-naïve than opioid-experienced humans and that repeated opioid exposure in pain patients for as few as 5 days can increase risk of long-term opioid use (Zacny et al., 1994; Shah et al., 2017). The present results extend these previous findings in two ways. First, initial morphine exposure produced primarily ICSS depression in both vehicle- and paclitaxel treated male rats, suggesting that initial expression of morphine reward was not enhanced by the paclitaxel-induced pain state. It has been argued that chronic pain states can enhance the rewarding effects of analgesic drugs by creating conditions under which those drugs produce negative reward (associated with reversal of an aversive pain state) in addition to whatever positive rewarding effects they may also produce (Navratilova et al., 2015). The present study did not find evidence for this phenomenon with morphine under conditions of a paclitaxel-induced neuropathic pain state in rats.

Second, paclitaxel also failed to alter the trajectory of increasing morphine reward produced by repeated morphine administration. This supports a previous study finding no effect of paclitaxel on morphine-induced conditioned place preference in rats (Mori et al., 2014) and extends on these studies by showing a failure of paclitaxel to alter the changes in morphine reward that occur in the ICSS procedure with a regimen of repeated morphine treatment. It is especially relevant to note that repeated morphine produced increasing expression of reward

while producing decreased expression of (i.e. tolerance to) anti-allodynia. This suggests a risk for iatrogenic addiction in use of morphine for treatment of CINP, as repeated treatment may produce a vicious cycle of analgesic tolerance and dose escalation that may simultaneously produce increasing sensitivity to morphine reward.

Finally, the present study found that initial morphine exposure produced stronger rewarding effects in vehicle- and paclitaxel-treated female rats than male rats, but repeated morphine exposure eliminated this sex difference and produced the same pattern of increasing reward despite anti-allodynic tolerance. These data agree with other rodent studies to suggest that females are more sensitive than males to abuse-related effects of mu opioid agonists (Craft, 2008). In self-administration studies, females acquired behavior for heroin and morphine faster than males (Cicero et al., 2003; Lynch, 2006; Lynch et al., 2013), and in a conditioned place preference study, females expressed greater place preference for high doses of morphine (Cicero et al., 2000). Due to the ability of ICSS procedures to detect both the abuse-limiting effects of drugs (e.g. motor depression causing decreases in rate reinforcement) as well as abuse-related facilitation, the lack of expression of abuse-related facilitation in morphine-naïve males may be related to previous observations of increased sensitivity of males to the sedative effects of opioids as opioids are more potent to suppress locomotion in males compared to females (Holtman et al., 2004; Craft et al., 2006). These preclinical findings in rodents map onto the few studies that have looked at the progression of opioid abuse in humans and are important because paclitaxel is often used to treat cancer in women while morphine is used to treat CINP in both women and men. In particular, women have an earlier age of initiation of opioid substance abuse and a more rapid progression from initial use to dependence (Anglin et al., 1987; Hernandez-

Avila et al., 2004) despite no large differences in overall prevalence of opioid use disorder (Becker et al., 2008; Manubay et al., 2015; Graziani and Nistico, 2016; Serdarevic et al., 2017).

Limitations

There were several limitations with the studies conducted and discussed in this dissertation. It is possible that the dose, the dosing regimens, and/or dosing intervals were not sufficient to induce a severe pain state that could reliably depress motivated behavior in the time frame tested. Increasing the dose produced severe weight loss or death. However, if injections were more spread out and given more than four times, a higher cumulative dose of chemotherapy could be achieved and might be sufficient to depress operant behavior reinforced by either brain stimulation or food pellets. An extension of this limitation is the time frame that behavioral depression was assessed. There is some evidence that depression of behavior manifests more intensely at later timepoints. In the studies described here, Day 29, the last time point often assessed often showed the most severe behavioral decreases outside of the injection window. Assessing timepoints later than those described here may also improve the sensitivity of these assays to detect chemotherapy-induced behavioral depression.

In the studies of Chapter II, individual variability was explored for paclitaxel-induced depression of ICSS. 26% of rats treated with either repeated 2.0 or 6.0 mg/kg paclitaxel had ICSS rates below those of the lowest saline-treated rat and could be considered depressed. This percentage is not far off from the 30% rate CINP following paclitaxel treatment in patients (Lavoie Smith et al., 2011). One implication of this could be that there are genetic factors that could predispose certain subjects to CINP. Sprague-Dawley rats, an outbred strain, were used in this study. While there is some genetic diversity within this strain of rats, the human population has much higher rates of genetic diversity. Utilization of additional strains of rats could help

explore the possible role of genetics in the predisposition to CINP. While currently outside of the capabilities of this author, genetic analysis of rats considered to be "responders" or that exhibit behavioral depression with paclitaxel could elucidate some of the potential mechanisms for chemotherapy-induced neuropathy. Lastly, one of the biggest limitations of this study and within the field studying CINP and potential analgesics to provide relief from CINP is that there is no effective treatment for this pain condition. A consequence of this preclinically, is that there is no positive control to compare the effectiveness of a candidate drug to reverse chemotherapy-induced behavioral depression. The two best drugs in terms of numbers needed to treat for neuropathic pain are morphine and nortriptyline (Finnerup et al., 2015). Both of these drugs were tested on chemotherapy-induced behavioral depression but failed to restore behavior to saline-treated levels.

Future Studies

Studies conducted here do not support the hypothesis that preclinical chemotherapy administration significantly alters motivated behaviors through decreasing mesolimbic dopamine signaling. This could be confirmed using microdialysis to measure extracellular dopamine levels in rats treated with vehicle and paclitaxel. It is possible that repeated cycles of chemotherapy may produce behavioral depression, and studies that achieve greater cumulative chemotherapy doses may produce a strong enough insult to detect these effects. However, in preliminary studies, a second cycle of 4 x 2.0 mg/kg paclitaxel treatment did not further depress ICSS reinforcements. It is also possible that surgical neuropathic manipulations such as spinal nerve ligation (SNL), chronic constriction injury (CCI), or partial sciatic nerve ligation (pSNL) may be strong enough insults to detect behavioral depressant effects; however studies in other laboratories do not support that hypothesis (Ewan and Martin, 2011a; Ewan and Martin, 2011b).

Studies described in Chapter III demonstrated significant disruptions of proprioception and/or motor competence with paclitaxel treatment in an assay of balance-beam performance. Future studies could investigate the effects of candidate neuroprotective drugs to reverse or prevent these deficits. Lastly, some studies have presented evidence to suggest that normally innocuous thermal or mechanical stimuli can become effective as punishers of operant responding in chemotherapy treated subjects (Mustafa et al., 2013; Abd-Elsayed et al., 2015; Ling et al., 2017). This type of experimental design could be useful to examine effects of candidate analgesics on chemotherapy-induced thermal or mechanical hypersensitivity in an assay of pain-depressed behavior without the risk of false-positive motor-depressing effects. An experiment was attempted which placed a cold metal plate on the floor of the ICSS chamber in an effort to test the hypothesis that cold hypersensitivity might punish ICSS responding in paclitaxel-treated rats. Specifically, our hypothesis was that chemotherapy-treated rats might be less willing than vehicle-treated rats to stand on a cold plate in order to access the ICSS response lever, and that as a result, they might show depressed ICSS responding. However, no differences between vehicle- and paclitaxel-treated rats were observed, and the design did not allow the experimenter enough control over the potential noxious stimulus (cold) to fully test this hypothesis.

Final Recommendations

One of the most striking inferences that can be made from the studies described in this dissertation is the incongruence between the endpoints of paclitaxel effects on mechanical sensitivity and operant behavior. Chemotherapy-induced mechanical hypersensitivity did not correlate with chemotherapy-induced decreases in ICSS, food-maintained responding, or body weight (but did correlate with decreases in IENFs). In addition, morphine was able to reverse chemotherapy-induced mechanical hypersensitivity but not chemotherapy-induced behavioral

depression, further evidence that these endpoints may be determined by different mechanisms. The need for a model of neuropathy-induced and pain-related behavioral depression to complement conventional assays of neuropathy-induced hypersensitive withdrawal responses cannot be overstated for the successful translation of drugs to alleviate CINP. Although that model was not uncovered in this dissertation, it is important to not rely on withdrawal responses as a sole endpoint in CINP drug development and to include sound experimental design to exclude motor depressing effects that make drugs look successful in attenuating withdrawal responses.

While the effects of neuropathy on the acute rewarding effects have been mixed, the work in this dissertation demonstrates the development of antinoceptive tolerance and enhancement of the rewarding effects of morphine regardless of the presence or absence of chemotherapy-induced neuropathy. Physicians treating CINP should be wary of the possible low effectiveness of opioid analgesics to relieve this pain state in conjunction with potential for development of analgesic tolerance and are cautioned to reconsider increasing the dose of opioid analgesics as the risk for abuse and addiction may increase with augmented and prolonged exposure to opiates.

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