BEHAVIORAL PHARMACOLOGICAL ANALYSIS OF DEEP BRAIN STIMULATION FOLLOWING NERVE INJURY

By

ERIC EDWARD EWAN

A Dissertation Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL of ARTS and SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Physiology and Pharmacology
December 2011

Winston-Salem, North Carolina

Approved by:
Thomas J. Martin, Ph.D., Advisor

Examinining Committee:
James C. Eisenach, M.D., Chairman

Steven R. Childers, Ph.D.
Robert C. Coghill, Ph.D.
Richard L. Rauck, M.D.
David C. Roberts, Ph.D.
Dedication

This work is dedicated to my family. Thank you for your continued support throughout my educational career, which has included moving halfway across the country for graduate school. Throughout this long journey, my family never tried to dissuade me from pursuing my goals, and instead provided a support system that has allowed me to pursue and obtain both personal and professional goals.

This work is especially dedicated to my wife and best friend, Lindsay. Though it was especially difficult for her to relocate away from family and friends, she has never wavered in her support of my decision to pursue a doctorate. Throughout this pursuit she has always been there, in good times and bad, and has helped to keep me focused throughout my time as a graduate student. Many times along this journey she has undoubtedly put my needs ahead of her own, and for that I am so very grateful.
ACKNOWLEDGEMENTS

I would first like to extend my gratitude to my advisor, Jeff Martin, for his support, patience, and guidance throughout my time in his lab. Jeff allowed me a great deal of latitude in pursuing my own research interests, as well as providing me with guidance in the planning and execution of experiments that collectively have culminated in this dissertation.

I would also like to thank my committee members: Steve Childers, Bob Coghill, Jim Eisenach, Rick Rauck, and Dave Roberts. Their key insights have significantly shaped this project and elevated its overall status. In addition, I would like to acknowledge the role of all members of the pain mechanisms lab, which is directed by Jim Eisenach. The weekly seminars, pain interest group meetings, and postdoc journal club all played a critical role in my scientific development. Of those members of the pain mechanisms lab, a special thanks to Chris Peters for the many casual scientific discussions, which were very helpful in my growth as a scientist.

Thank you to current and former members of the Martin lab, Susy Kim and Nancy Buechler, for your assistance over the course of my time in the lab. I would also like to acknowledge the National Institute on Drug Abuse, whose financial support made this work possible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER I</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>CHAPTER II</td>
<td>35</td>
</tr>
<tr>
<td>OPIOID FACILITATION OF REWARDING ELECTRICAL BRAIN STIMULATION IS SUPPRESSED IN RATS WITH NEUROPATHIC PAIN</td>
<td></td>
</tr>
<tr>
<td>Published in Anesthesiology, 2011</td>
<td></td>
</tr>
<tr>
<td>CHAPTER III</td>
<td>73</td>
</tr>
<tr>
<td>REWARDING ELECTRICAL BRAIN STIMULATION IN RATS FOLLOWING PERIPHERAL NERVE INJURY: DECREASED FACILITATION BY COMMONLY ABUSED PRESCRIPTION OPIOIDS</td>
<td></td>
</tr>
<tr>
<td>Accepted in Anesthesiology 2011</td>
<td></td>
</tr>
<tr>
<td>CHAPTER IV</td>
<td>114</td>
</tr>
<tr>
<td>SUPPRESSION OF REWARDING BRAIN STIMULATION AFTER PAW INCISION IN RATS</td>
<td></td>
</tr>
<tr>
<td>CHAPTER V</td>
<td>139</td>
</tr>
<tr>
<td>INTRACRANIAL SELF-STIMULATION OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS: EVIDENCE FOR OXYTOCIN-MEDIATED REINFORCEMENT MECHANISMS</td>
<td></td>
</tr>
<tr>
<td>Submitted to Anesthesiology</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER VI. ............................................................... 173

INTRACRANIAL SELF-STIMULATION OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS: INCREASED FACILITATION BY MORPHINE COMPARED TO COCAINE

Submitted to Anesthesiology

CHAPTER VII .............................................................. 201

ELECTRICAL STIMULATION OF THE PERIAQUEDUCTAL GRAY FAILS TO ELICIT OPERANT BEHAVIOR IN NERVE-INJURED RATS

CHAPTER VIII ............................................................. 220

DISCUSSION

CURRICULUM VITA ...................................................... 236
**List of Illustrations**

### Chapter 1

**Figure 1.** Descending pain modulation circuitry ........................................ 6

**Figure 2.** Amygdala involvement in pain and limbic-reward pathways ........... 11

### Chapter 2

**Figure 1.** Schematic showing the location of stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats .......................................................... 47

**Figure 2.** Development of mechanical allodynia following spinal nerve ligation (SNL) and baseline responding for electrical stimulation of the ventral tegmental area in control and SNL rats. ............... 50

**Figure 3.** Effects of spinal nerve ligation (SNL) on morphine facilitation of electrical stimulation of the ventral tegmental area for drug incubation times of 15-min (A) and 60-min (B) .................. 53

**Figure 4.** Effects of spinal nerve ligation (SNL) on heroin facilitation of electrical stimulation of the ventral tegmental area .................. 55

**Figure 5.** Effects of spinal nerve ligation (SNL) on cocaine facilitation of electrical stimulation of the ventral tegmental area .................. 58

**Figure 6.** Drug effects on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats .................. 60

### Chapter 3

**Figure 1.** Location of the stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats .................. 87

**Figure 2.** Baseline responding for electrical stimulation of the ventral tegmental area (VTA ICSS) and the effects of 3mg/kg morphine facilitation of VTA ICSS in control and spinal nerve ligated (SNL) rats. .................. 90

**Figure 3.** Effects of spinal nerve ligation (SNL) on fentanyl facilitation of electrical stimulation of the ventral tegmental area .................. 93
Figure 4. Effects of spinal nerve ligation (SNL) on methadone (A) hydromorphone (B), and oxycodone (C) facilitation of electrical stimulation of the ventral tegmental area. 95

Figure 5. Opioid effects on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. 98

Figure 6. Effects of spinal analgesics on facilitation of electrical stimulation of the ventral tegmental area and on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. 101

Table 1 Effects of electrical stimulation of the ventral tegmental area on mechanical allodynia. 103

Chapter 4

Figure 1. Schematic showing the location of stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats. 124

Figure 2. Mechanical hypersensitivity around the incision site following paw incision in control and spinal nerve-ligated (SNL) rats. 127

Figure 3. Effects of paw incision on responding for electrical stimulation of the ventral tegmental area (VTA ICSS) in control and spinal nerve-ligated (SNL) rats. 129

Figure 4. Effects of paw incision on responding for electrical stimulation of the ventral tegmental area (VTA ICSS) in control and spinal nerve-ligated (SNL) rats. 132

Chapter 5

Figure 1. Location of the stimulating electrodes within the paraventricular nucleus of the hypothalamus and medial forebrain bundle for control and spinal nerve-ligated (SNL) rats. 150

Figure 2. Effects of electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. 154

Table 1 Effects of electrical stimulation of the medial forebrain bundle on mechanical allodynia. 155
Figure 3. Baseline responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. .......................... 158

Figure 4. Effects of intrathecal drugs on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. ......................................................... 160

Figure 5. Effects of intraperitoneal atosiban and naltrexone on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. .................. 163

Chapter 6

Figure 1. Location of the stimulating electrodes within the paraventricular nucleus of the hypothalamus and medial forebrain bundle for control and spinal nerve-ligated (SNL) rats ......................... 182

Figure 2. Baseline responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. .............. 186

Figure 3. Effects of morphine on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. ................................................................. 189

Figure 4. Effects of cocaine on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. ................................................................. 192

Chapter 7

Figure 1. Location of the stimulating electrodes within the periaqueductal Gray in spinal nerve-ligated (SNL) rats ......................... 210

Figure 2. Effects of electrical stimulation of the periaqueductal grey (PAG) on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats ................................................................. 213
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Amygdala</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned Place Preference</td>
</tr>
<tr>
<td>DA</td>
<td>Dopaminergic</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
</tr>
<tr>
<td>EF50</td>
<td>Frequency Maintaining 50% Maximal Responding</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamatergic</td>
</tr>
<tr>
<td>ICSS</td>
<td>Intracranial Self-Stimulation</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>i.t.</td>
<td>Intrathecal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial Forebrain Bundle</td>
</tr>
<tr>
<td>MOR</td>
<td>Mu Opioid Receptor</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial Prefrontal Cortex</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus Raphe Magnus</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal Grey</td>
</tr>
<tr>
<td>PBN</td>
<td>Parabrachial Nucleus</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus of the Hypothalamus</td>
</tr>
<tr>
<td>PWT</td>
<td>Paw Withdrawal Threshold</td>
</tr>
<tr>
<td>Rmax</td>
<td>Maximal Response Rate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral Ventral Medulla</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal Nerve Ligation</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
</tbody>
</table>
ABSTRACT

Ewan, Eric Edward

BEHAVIORAL PHARMACOLOGICAL ANALYSIS OF DEEP BRAIN STIMULATION FOLLOWING NERVE INJURY

Dissertation under the direction of
Thomas J. Martin, PhD., Associate Professor of Anesthesiology

Treatment of neuropathic pain remains a major unmet public health problem. Serious concerns arise over misuse of prescription opioids in chronic pain patients, a drug class representing the fastest growing substance abuse problem in the United States. Ascending pain pathways overlap significantly with limbic regions involved in reward and reinforcement, therefore pain and reward systems substantially interact and modulate one another. The extent to which pain modulates mesolimbic dopamine systems and thereby alters the reinforcing and abuse-related effects of opioids is not fully understood. The ability of pain relief to produce reinforcement in and of itself through modulation of this circuitry is also unknown.

To address these concerns the current work employed intracranial self-stimulation (ICSS), an operant paradigm pairing lever presses with electrical stimulation of discrete brain regions. Studies employing ventral tegmental area (VTA) ICSS in normal and neuropathic rats suggest that mesolimbic dopamine neurons function normally after nerve injury, yet stimulation of these pathways by opioids appears to be selectively diminished. In addition, alleviation of hypersensitivity (and possibly spontaneous pain) with spinal analgesics does not
stimulate this circuitry following neuropathy. In contrast, acute pain induced by paw incision produces brief suppression of mesolimbic dopamine pathways, suggesting that intense spontaneous pain in the postoperative period, but not pain following neuropathy, inhibits limbic reward pathways in rats.

Stimulation of the hypothalamic paraventricular nucleus (PVN) and periaqueductal gray (PAG) reversed mechanical hypersensitivity induced by spinal nerve ligation, though only PVN stimulation produced reinforcing effects in neuropathic rats. PVN stimulation also produced reinforcing effects in normal rats, and the reinforcing effects of PVN ICSS in neuropathic rats were not altered by spinal analgesics. These studies suggest that alleviation of hypersensitivity is unlikely to be the primary stimulus mediating ICSS in the PVN.

Collectively, these studies provide some insights into the interactions between pain and reward systems, and how each is altered under neuropathic states. These studies also highlight the difficulties in assessing spontaneous pain in laboratory animals. Given the results from these studies, it is unclear if nerve-injured rats experience significant spontaneous pain after peripheral nerve injury, or if the ICSS paradigm as it is typically used is not sensitive enough to detect subtle manifestations of ongoing pain in neuropathic rats. Future studies utilizing different approaches in performing ICSS studies, as well as studying the effects of different nerve injury models or other chronic pain models may be useful in addressing these issues.
Chapter I

Introduction
Clinical Significance

Chronic neuropathic pain is debilitating and leads to a diminished health-related quality of life (Meyer-Rosberg et al., 2001). Treatment with analgesics, including opioids, often provides insufficient pain relief (Orza, Boswell, & Rosenberg, 2000). Furthermore, major concerns over the chronic use of prescription opioids arise due to concerns over tolerance, side effects, and abuse (Compton & Volkow, 2006). Prescription opioid abuse is the fastest growing drug abuse problem in the United States (SAMHSA 2008). Given the high incidence of chronic pain in the United States, with some estimates ranging between 10-20% (Rosenblum, Marsch, Joseph, & Portenoy, 2008), and the dramatic increase in written prescriptions of opioids in recent years (Zacny et al., 2003), a clear need exists for examining the interaction between pain and abuse liability of prescription opioids. Ultimately, the goal of such an endeavor will be to identify compounds with high analgesic efficacy but with limited abuse liability.

One major concern regarding translation of preclinical pain research to the clinic is the dependent measures typically used in preclinical studies. Interest in moving beyond simple reflexive measures, which have more recently been criticized for their clinical relevance, has led to interest in measuring spontaneous pain in the absence of an overt noxious stimulus. Mogil and Crager (2004) have commented extensively on this topic, providing the rationale that “if the mechanisms underlying different hypersensitivity states are dissociable… why would we assume that the mechanisms underlying spontaneous pain are the same as those underlying any form of hypersensitivity?” These authors continue
to describe the challenges of such a pursuit, stating that “we do not have an immediate solution to this problem, because we also cannot point to a behavior (or set of behaviors) that sensitively, uniquely and reliably indicate the existence and relative intensity of spontaneous chronic pain.” In the pursuit of identifying relevant measures of spontaneous pain, it is possible that efforts will be repeatedly met with disappointing and unsuccessful results. The possibility of identifying a behavioral measure that is sensitive to spontaneous pain in animals may be well worth it, and may enhance the clinical translation of preclinical pain studies.

**Background: Pain Physiology**

Pain is detected in the periphery by specialized nociceptors that respond to noxious stimulation and transmit pain signals to the spinal cord via primary afferents. These afferent fibers include myelinated A\(\delta\) and unmyelinated C fibers that transmit fast and slow nociception, respectively (D'Mello & Dickenson, 2008). Noxious input from A\(\delta\) and C fibers mostly terminate in the superficial laminae of the spinal cord (Todd, 2002), where they synapse with ascending projection neurons that terminate in many different supraspinal regions (Almeida, Roizenblatt, & Tufik, 2004). The site of supraspinal termination of pain pathways is critical in determining the consequences of noxious stimuli. From this viewpoint, pain is comprised of both sensory and affective dimensions. The spinothalamic pathway is implicated in both sensory and affective dimensions of pain. Spinothalamic projections to the lateral thalamus synapse onto tertiary
neurons that in turn project to the somatosensory cortex which signal the type and location of pain (Hunt & Mantyh, 2001). Projections to the medial thalamus however synapse on neurons that in turn project to the anterior cingulate cortex (Rainville, Duncan, Price, Carrier, & Bushnell, 1997), which is implicated in encoding affective responses to pain. The other predominant nociceptive pathway implicated in the affective dimension of pain is the spinoparabrachial pathway, which arises from spinal projections to the parabrachial area and the periaqueductal grey (PAG); these areas in turn project to the hypothalamus and amygdala, which are thought to be critical in signaling the intensity and emotional responses to pain (Hunt & Mantyh, 2001).

The brain has a tremendous capacity to modulate incoming nociceptive input. Much of this is accomplished by activation of descending pain pathways that modulate spinal pain transmission. One of the most well described descending pain modulating system consists of the amygdala, PAG, and rostroventral medulla (RVM). (Figure 1) The central amygdala (CeA) sends GABAergic projections to the PAG (Swanson & Petrovich, 1998) which in turn sends excitatory output to the RVM (Basbaum & Fields, 1984). The RVM in turn projects to pain transmitting neurons in the spinal cord to either enhance (via RVM ON cells) or inhibit (via RVM OFF cells) ascending spinal pain transmission (Fields, 2000). Importantly, many therapeutic strategies to treat pain attempt to enhance descending inhibition or diminish descending facilitation, making elucidation of the underlying circuitry of both an important research venture.
Figure 1. Descending pain modulation circuitry. The basolateral amygdala (BLA) receives noxious input from cortical and ascending parabrachial neurons. GABAergic projections from the BLA synapse in the central amygdala (CeA). GABAergic projections from the CeA synapse in the periaqueductal gray (PAG), which in turn send excitatory output to the rostroventral medulla (RVM). RVM ON cells enhance firing of dorsal horn neurons and stimulate pain transmission, whereas RVM OFF cells conversely inhibit firing of dorsal horn neurons and inhibit spinal pain transmission. Opioids inhibit ON cells and stimulate OFF cells, which likely mediates some of their analgesic effects.
Animal models that simulate clinical neuropathies have been developed to study the pathophysiology of neuropathic pain (Martin & Eisenach, 2001). This has led to a wealth of knowledge regarding peripheral and central mechanisms contributing to neuropathic pain. Peripheral sensitization refers to increased spontaneous activity and responsiveness to future stimulation of nociceptors following nerve injury. For instance, nerve injury produces an immediate discharge from the injured afferent nerve (Wall, Waxman, & Basbaum, 1974) as well as long-lasting spontaneous activity of both injured and non-injured fibers (Ali et al., 1999). At the same time, the damaged fibers release a number of inflammatory mediators that sensitize nociceptors (Ji & Strichartz, 2004) and subsequently reduce their thresholds to transmit nociception.

Central sensitization results as a consequence of peripheral sensitization following nerve injury. The dramatic increase in noxious input to the spinal cord induces the release of a number of pain enhancing substances, such as the excitatory neurotransmitters glutamate and aspartate as well as the neuromodulator substance P, in the spinal cord dorsal horn (Holden & Pizzi, 2003); each of these substances function to facilitate pain transduction to second order projection neurons. Additionally, glial cells become activated following nerve injury (Watkins, Milligan, & Maier, 2001) and subsequently release additional substances to those mentioned, including prostaglandins and growth factors, that further facilitate spinal pain transmission (Watkins & Maier, 2000). In addition to the enhanced excitatory drive, reductions in spinal immunoreactivity for GABA have been shown following nerve injury (Ibuki, Hama, Wang, Pappas,
& Sagen, 1997), suggesting that loss of spinal inhibitory tone also contributes to central sensitization. Furthermore, alterations of descending pain modulation circuitry have been implicated in contributing to central sensitization, in particular increased descending pain facilitation from the RVM following nerve injury (Kovelowski et al., 2000). To this end nerve injury produces spinal and supraspinal plasticity resulting in increased excitatory and decreased inhibitory tone within the spinal cord, which collectively results in profound facilitation of pain transmission.

**Interactions between Pain and Reward Circuits**

The amygdala is a midbrain region that plays a critical role in mediating interactions between pain pathways and the limbic system. The amygdala receives direct nociceptive input from the parabrachial nucleus (Bernard & Besson, 1990). Activation of the amygdala occurs during a number of pain states and correlates with pain behaviors in rats, and pharmacological inhibition of the amygdala suppresses many of these pain related behaviors (Neugebauer, Galhardo, Maione, & Mackey, 2009). The amygdala can also indirectly modulate spinal pain transmission via descending projections to the PAG (Figure 1). Local administration of the mu opioid receptor (MOR) agonist DAMGO into the BLA increases tail flick latency to noxious heat that is blocked by pharmacological inhibition of the PAG or RVM (Helmstetter, Tershner, Poore, & Bellgowan, 1998), indicating that antinociception produced by amygdala opioid receptor activation is mediated through these descending pathways.
The amygdala also plays a role in reward circuitry. Neurons in the ventral tegmental area (VTA) send dopaminergic projections to the nucleus accumbens (NAcc), medial prefrontal cortex (mPFC), ventral pallidum, hippocampus, and amygdala (Pierce & Kumaresan, 2006) (Figure 2), although most work has focused on dopamine release in the NAcc as the major neural substrate for reward. The amygdala modulates activity of this circuit via GABAergic projections from the CeA that synapse on dopaminergic cell bodies of the VTA (Everitt et al., 1999) as well as via glutamatergic projections from the BLA that synapse on dopaminergic nerve endings in the NA and mPFC (Brog, Salyapongse, Deutch, & Zahm, 1993; Wright, Beijer, & Groeneweegen, 1996) (Figure 2). Consequently, the amygdala can inhibit or enhance limbic dopamine neurotransmission.

Evidence suggests that pain suppresses mesolimbic dopamine, as well as its modulation by opioids. Phosphorylation of extracellular signal-regulated kinase in tyrosine hydroxylase-positive cells of the VTA is suppressed following nerve injury in mice (Ozaki, Narita, Ozaki, Khotib, & Suzuki, 2004), suggesting reduced activity of VTA dopamine neurons following neuropathy. Similarly, nerve injury reduces the rewarding effects of morphine assessed using the conditioned place preference (CPP) paradigm, and is associated with reductions in morphine-stimulated GTPγS in the VTA and morphine-induced dopamine release in the NA in rats (Ozaki et al., 2002). Similar observations have been made in mice (Ozaki, Narita, Iino, Miyoshi, & Suzuki, 2003).

The mechanism(s) by which pain suppresses this circuitry is unclear. One possibility is that noxious input to the parabrachial nucleus (PBN), which projects
Figure 2. Amygdala involvement in pain and limbic-reward pathways. The basolateral amygdala (BLA) receives nociceptive glutamatergic (Glu) input from the somatosensory cortex and parabrachial nucleus (Pb Nuc). Glutamatergic fibers from the BLA project to limbic forebrain regions, including the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), and synapse directly on ascending dopaminergic (DA) nerve endings originating in the ventral tegmental area (VTA). GABAergic projections from the BLA synapse in the central amygdala (CeA), which in turn sends GABAergic efferents that synapse on dopamine cell bodies in the VTA.
to the VTA, may directly inhibit VTA dopamine neurons. For instance, administration of lidocaine into the PBN suppresses foot shock-induced inhibition of VTA dopamine neurons (Coizet, Dommett, Klop, Redgrave, & Overton, 2010). Therefore, increased PBN input following nerve injury may suppress VTA dopamine neurons. Similarly, the CeA is activated during pain states, and it is possible that increased firing of GABAergic neurons from the CeA to the VTA suppresses dopaminergic cell bodies in the VTA in neuropathic rats. Clinically, suppression of this circuitry may underlie the comorbidity of chronic pain with affective disorders such as anxiety and depression.

The extent to which activation of mesolimbic dopamine alters pain processing is poorly understood. The VTA contains mu opioid receptors located predominantly on non-dopaminergic cells (Garzon & Pickel, 2001) and activation of these receptors disinhibits dopamine cell bodies within the VTA, which is thought to be critical in mediating the reinforcing effects of morphine microinjected into the VTA (Bozarth & Wise, 1981). Direct injection of morphine into the VTA (Manning, Morgan, & Franklin, 1994) alleviates ongoing pain behaviors following formalin injection into the hindpaw, an effect that is reversed by pretreatment with intra-NA dopamine receptor antagonists (Altier & Stewart, 1998). Interestingly, tail-flick latency to noxious heat is unaltered following intra-VTA morphine, suggesting that mesolimbic dopamine modulates behavioral responses to persistent pain but not spinally mediated transient pain (Altier & Stewart, 1999). It is possible that these differential effects of intra-VTA morphine may be due to affective analgesia (Franklin, 1989), which predicts that
activation of the dopamine systems alters affect to such a degree that the animal becomes indifferent to nociceptive input. Therefore, opioids may to some degree function as analgesics by altering one’s affective state through actions within the mesolimbic dopaminergic system.

**Measuring Neuropathic Pain in Animals**

Following peripheral nerve injury, animals develop hypersensitivities to external stimuli and exhibit behaviors thought to be indicative of ongoing spontaneous pain (Mogil & Crager, 2004). Of these behaviors, the most frequently studied is hindpaw hypersensitivity, and in many cases it is the sole behavioral endpoint. This may be problematic since many compounds that alleviate hypersensitivity in animals have failed clinical trials (Mogil & Crager, 2004). This may be because the chief complaint of neuropathic pain patients is ongoing spontaneous pain (Backonja & Stacey, 2004) which may be unrelated to hypersensitivity (Gottrup, Nielsen, Arendt-Nielsen, & Jensen, 1998). This has increased interest in preclinical development of behavioral endpoints designed to assess spontaneous pain in animals.

Many behavioral measures are suggested to be indicative of spontaneous pain, yet no consensus exists on how to measure spontaneous pain in animals. Recent work with drug self-administration and conditioning experiments lends support for utilization of these methodologies to assess spontaneous pain in animals. Administration of intrathecal (i.t.) clonidine selectively reduces intravenous (i.v.) heroin self-administration (Martin, Kim, Buechler, Porreca, &
Eisenach, 2007) and selectively produces conditioned place preference (CPP) (King et al., 2009) in nerve-injured rats. Interestingly, i.t. adenosine did not produce similar effects as clonidine in each of these studies, although both drugs alleviated established mechanical allodynia. In humans with neuropathic pain both clonidine and adenosine alleviate allodynia, but only clonidine alleviates spontaneous pain (Eisenach, DuPen, Dubois, Miguel, & Allin, 1995; Eisenach, Rauck, & Curry, 2003), suggesting that the selective effect of clonidine in nerve-injured rats may be due to alleviation of spontaneous pain. This suggests that these assays are sensitive to therapies that alleviate spontaneous pain, and suggests that alleviation of hypersensitivity can be achieved without altering spontaneous pain in animals.

Operant paradigms in rodents such as drug self-administration have considerable face validity with regards to human analgesic use. The frontal lobe is critical in cognitive functions such as decision-making and goal-directed behaviors exhibited by rodents performing operant tasks (Vertes, 2006), and is also interconnected with limbic regions critical in signaling pain intensity (Hunt & Mantyh, 2001; McDonald, 1991). Therefore, manipulations to the somatosensory system (e.g., analgesics) which alter complex operant behavior likely do so by altering activity within limbic-cortical pathways. To date, few pain studies in animals utilize complex operant behavior as a dependent measure. The major advantages of using an operant paradigm to study pain versus exclusive reliance on reflexive measures are threefold. First, operant tasks can be performed in the absence of an external noxious stimulus, allowing for the assessment of ongoing
spontaneous pain. Second, operant behavior resulting in delivery of a potential therapeutic (pharmacological or other) inherently infers some degree of subject compliance. Third, operant behavior requires decision-making that is mediated to some extent by limbic-cortical connections (Vertes, 2006), therefore providing information regarding the motivational-affective state of the animal performing the operant task.

**Deep Brain Stimulation (DBS)**

Electrical stimulation of discrete brain regions has proven successful in some cases in the treatment of drug-resistant pain. Initial clinical success was reported with stimulation of the thalamus and PAG (Gybels & Kupers, 1990), and more recently has been shown with motor cortex stimulation (Carroll et al., 2000). In many cases, however, the precise mechanism(s) underlying stimulation induced analgesia remain unclear.

Preclinical studies have provided some evidence for the underlying mechanism(s) associated with DBS. Interest was initially sparked by the revelation that rats could endure painful laparotomies in the absence of anesthesia during stimulation of the PAG (Reynolds, 1969). Further studies assessing the effects of DBS on multiple brain sites reveal that stimulation of the PAG produces the most consistent and robust analgesic effects compared to other brain sites (Mayer & Liebeskind, 1974), supporting its use as a clinical target. Additional studies have reported that stimulation of the dorsal PAG is associated with aversive behavior (Oliveras & Besson, 1988) whereas
stimulation of the ventral PAG produces analgesia in rats (Fardin, Oliveras, & Besson, 1984).

The analgesic effects of PAG stimulation are likely mediated by activation of descending projections to the RVM which in turn inhibit spinal pain processing (Basbaum & Fields, 1984). PAG stimulation suppresses the activity of dorsal horn neurons, which is associated with spinal release of serotonin and norepinephrine (Cui, Feng, McAdoo, & Willis, 1999); thus PAG stimulation may indirectly stimulate descending projections of the dorsal raphe magnus or locus coeruleus to inhibit spinal pain transmission. It is well known that opioids exert analgesia through the PAG-RVM circuitry (Fields, 2000), and it has been suggested for decades that opioids and DBS of the PAG share similar mechanisms (Mayer & Liebeskind, 1974); such a conclusion is supported by the fact that stimulation induced analgesia of the PAG is reversed by the opioid antagonist naloxone in both normal (Akil, Mayer, & Liebeskind, 1976) and nerve-injured rats (Lee, Park, Won, Park, & Sohn, 2000).

Animal studies also support the clinical use of thalamic stimulation for pain relief. A study in nerve-injured rats revealed that stimulation of the ventroposterolateral thalamus produces a slow onset alleviation of mechanical allodynia that peaks after 20 minutes of stimulation and slowly returns to baseline once the stimulation is turned off (Kupers & Gybels, 1993). Mechanisms of thalamic stimulation induced analgesia are even less clear than PAG. Thalamic stimulation reduces activity of spinothalamic tract neurons in the dorsal horn of the spinal cord in both rats (Dickenson, 1983) and primates (Gerhart, Yezierski,
Fang, & Willis, 1983), although no direct spinal projections from the thalamus have been documented. One possibility is that antidromic stimulation of ascending spinothalamic fibers, particularly collaterals of this pathway to the PAG and NRM (Giesler, Yezierski, Gerhart, & Willis, 1981) may activate descending pain modulatory circuits and therefore indirectly inhibit spinal pain transmission; this potential mechanism would explain why NRM lesions block thalamic stimulation-induced inhibition of dorsal horn spinothalamic neurons in rats (Dickenson, 1983). Additionally, thalamic stimulation may be related to classic gate control theory (Melzack & Wall, 1965), which predicts analgesia by low threshold stimulation of ascending non-nociceptive fibers, since clinically thalamic stimulation is associated with an increase in regional changes in blood flow in the ipsilateral somatosensory cortex (Duncan et al., 1998).

A preclinical brain region gaining increased interest is the hypothalamic paraventricular nucleus (PVN), which plays a pivotal role in neuroendocrinology and endogenous analgesia. The two main neurosecretory systems include the magnocellular and parvocellular neurons that project from the PVN. Magnocellular neurons synthesize and release oxytocin and vasopressin into the posterior pituitary, where they are stored and released into the bloodstream in response to physiologic stimuli. Parvocellular neurons project to other regions of the CNS, particularly the median eminence, where they secrete hormones that stimulate the release of anterior pituitary hormones. For instance, activation of the hypothalamic-pituitary-adrenal axis in response to stress is an important function of parvocellular neurons (Herman, Prewitt, & Cullinan, 1996).
In addition to its major interaction with the pituitary gland, the PVN also sends central oxytocin projections to a number of targets including the forebrain, limbic system, brainstem, and spinal cord (Gimpel & Fahrenholz, 2001) that have been implicated in the analgesic effects of oxytocin. Oxytocin receptors are located throughout the CNS, and are located in close proximity to oxytocinergic nerve endings (Viero et al., 2010). Electrical stimulation of the PVN causes a substantial release of oxytocin in CSF, plasma, and the spinal cord, and partially reverses established allodynia following nerve injury that is reversed with spinal delivery of an oxytocin receptor antagonist (Martinez-Lorenzana et al., 2008). Additionally, oxytocin has been shown to produce antinociception in rats following i.p. (Lundeberg, Uvnas-Moberg, Agren, & Bruzelius, 1994), intracerebroventricular (ICV; (Gao & Yu, 2004), and i.t. (Condes-Lara et al., 2006) administration. Further, analgesia produced by electrical stimulation of the PVN may be opioid mediated, since spinal naloxone partially reverses the antiallodynic effects of PVN stimulation (Condes-Lara, Rojas-Piloni, Martinez-Lorenzana, Lopez-Hidalgo, & Rodriguez-Jimenez, 2009). To this end, oxytocin release from the PVN has the potential to produce analgesia through a number of central and peripheral sites.

The PVN sends oxytocinergic fibers to regions within the limbic system and may modulate behavioral effects of drugs of abuse. These effects likely arise through release of oxytocin in limbic and forebrain regions, where oxytocin influences dopaminergic transmission (Baskerville & Douglas, 2010). For instance, s.c. and i.c.v. oxytocin administration has been shown to inhibit
tolerance to heroin (Kovacs, Borthaiser, & Telegdy, 1985) and morphine (Kovacs et al., 1987) antinociception in mice. Similarly, s.c. and i.c.v oxytocin has been shown to inhibit some of the acute behavioral effects of cocaine, such as cocaine-induced stereotypy (Sarnyai et al., 1991). Oxytocin may not only interact with drugs of abuse, but may exert rewarding properties in its own right through interactions with mesolimbic dopaminergic neurons. Oxytocinergic fibers project from the PVN to the VTA, and intra-VTA oxytocin results in dopamine release in the NAcc that is reversed by pretreatment with an intra-VTA oxytocin receptor antagonist (Melis et al., 2007). Interestingly, repeated s.c. administration of oxytocin produces conditioned place preference in rats (Liberzon, Trujillo, Akil, & Young, 1997), though it is unclear if this is due to effects on the limbic system.

Despite its success, DBS has been compromised by the fact that tolerance to the analgesic effect occurs frequently (Levy, Lamb, & Adams, 1987) as well as the occurrence of unwanted side effects. Pain research using DBS in animals suffers from similar pitfalls to those previously described. Most animal studies using DBS have assessed the effects of stimulation on altering thresholds to noxious stimuli in normal rats. Similarly, studies of DBS that have been conducted in neuropathic rats typically have only assessed stimulation effects on nerve injury-induced hypersensitivity as the sole dependent measure. A recent study reports that motor cortex stimulation produces CPP selectively in rats with spinal cord lesions (Davoody et al., 2011), suggesting that ongoing pain may be attenuated with DBS and may be reinforcing during chronic pain states in animals.
Intracranial Self-Stimulation (ICSS)

In the 1950’s it was discovered that electrical stimulation of discrete brain regions produces reinforcing effects in rats (Olds & Milner, 1954). Rats will perform operant behavior (e.g., lever press) to receive stimulation of many discrete brain regions (Phillips, 1984), and early studies suggested an important role for dopamine in contributing to the reinforcing effects of multiple brain sites (Cooper & Breese, 1975). In support of an important role of dopamine in the reinforcing effects of ICSS, brain regions supporting the most robust levels of ICSS lie within the mesolimbic dopamine system, including the VTA and medial forebrain bundle (MFB) (Wise, 1996), and studies of these regions comprise the bulk of the ICSS literature.

The reinforcing effects of ICSS of the VTA and MFB are intricately related to specific parameters of the electrical stimulation. Two of the most important parameters include the amount of current (μA) administered and the frequency of stimulation (Hz); these parameters determine the number of cells that are stimulated as well as the frequency at which these cells fire action potentials, respectively (Wise, Bauco, Carlezon, & Trojniar, 1992). Importantly, the reinforcing effects of ICSS are directly related to each of these parameters, such that higher intensities and frequencies of stimulation correspond to higher rates of responding.

The fact that ICSS is tightly controlled by stimulation parameters has led to the development of sophisticated techniques for assessing the reinforcing effects
of brain stimulation as well as its modulation by pharmacological and environmental manipulations. One of the most frequently used techniques to study ICSS is the curve-shift method (Miliaressis, Rompre, Laviolette, Philippe, & Coulombe, 1986). With this method, one parameter (e.g., current) is held constant meanwhile another parameter (e.g., frequency) is systematically altered, producing frequency-response curves for ICSS that mimic pharmacological dose-effect curves (Carlezon & Chartoff, 2007). Under these conditions manipulations that facilitate or suppress the reinforcing effects of ICSS can be assessed by their ability to produce leftward or rightward shifts to the frequency-response curve, respectively.

A number of studies provide strong support for dopamine-mediated reward associated with VTA or MFB ICSS. Electrical stimulation of the VTA (Fiorino, Coury, Fibiger, & Phillips, 1993) and MFB (You, Chen, & Wise, 2001) induces dopamine release in the NAc, and direct injection of a dopamine antagonist into the NAc suppresses responding for VTA ICSS when the injection is ipsilateral, but not contralateral, to the stimulation site (Mogenson, Takigawa, Robertson, & Wu, 1979). Similarly, 6-hydroxydopamine lesions of ascending dopamine fibers from the VTA suppresses responding for VTA ICSS, and this effect is selective for lesions that are ipsilateral to the stimulation site (Fibiger, LePiane, Jakubovic, & Phillips, 1987). Conversely, most drugs of abuse, such as dopamine and MOR agonists, facilitate responding for ICSS (Wise, 1996). Since the reinforcing effects of drugs of abuse are to some extent mediated by stimulation of mesolimbic dopamine (Pierce & Kumaresan, 2006), the fact that they facilitate
ICSS further supports the notion that ICSS is mediated by stimulation of mesolimbic dopamine, and suggests a shared neural substrate for the rewarding effects of ICSS and many drugs of abuse.

It seems to reason that since ICSS produces reinforcing effects mediated by dopamine release, and that brain regions within the mesolimbic dopamine system (VTA or MFB) produce the most robust responding for ICSS, that the effects are due to direct electrical stimulation of ascending dopamine neurons. However, electrophysiological studies suggest that direct electrical stimulation of non-dopaminergic cells is also involved. First, dopamine neurons have higher stimulation threshold requirements (e.g., current) than those typically used during ICSS sessions (Yeomans, Maidment, & Bunney, 1988), and therefore dopamine fibers likely comprise only a small portion of the fibers stimulated during VTA and MFB ICSS (Yeomans, 1989). Second, most fibers shown to be directly stimulated by MFB stimulation possess shorter refractory periods (Yeomans, 1979) and faster conduction velocities (Bielajew & Shizgal, 1982) than unmyelinated ascending dopamine neurons, which was assessed using paired-pulse and collision tests, respectively. Third, a two-electrode study revealed that many MFB stimulated fibers project in the rostral-caudal direction, since anodal hyperpolarization of the VTA suppressed the effects of MFB stimulation (Bielajew & Shizgal, 1986). Much evidence suggests that many of the stimulated rostral-caudal fibers are cholinergic and form a loop through the pedunculopontine tegmental nucleus that feeds back and stimulates VTA dopamine neurons via muscarinic receptors (Yeomans, Kofman, & McFarlane, 1985; Yeomans, Mathur,
Stimulation of glutamatergic fibers may also be important, since MFB stimulation induces glutamate release in the VTA (You et al., 2001), which would be expected to stimulate dopamine neurons via NMDA receptors (Chergui et al., 1993). To this end, stimulation of the VTA and MFB produces reinforcing effects by stimulating mesolimbic dopamine, which likely occurs through both direct and indirect stimulation of ascending dopamine pathways originating from the VTA that terminate in the NAc.

To date, few studies have examined the extent to which pain modulates the reinforcing effects of ICSS. A recent study indicates that acute pain produced by administration of intraperitoneal lactic acid, a noxious stimulus, suppresses responding for MFB ICSS (Pereira Do Carmo, Stevenson, Carlezon, & Negus, 2009). Since the reinforcing effects of ICSS are mediated by stimulation of mesolimbic dopamine, this suggests that an acute noxious stimulus suppresses this system, and suggests that the ICSS methodology is sensitive to pain manipulations. It is unclear if chronic pain induced by nerve injury will similarly diminish the reinforcing effects of ICSS, or if ongoing pain following nerve injury alters opioid pharmacology of ICSS.
Statement of Purpose

The ICSS paradigm provides a unique approach for studying interactions between pain and reward systems. Electrodes can specifically target brain regions implicated in reward, analgesia, or both, and the reinforcing effects of stimulation can be measured using operant behavior. Since ICSS studies are performed in the absence of an external noxious stimulus, this technique has the potential to measure effects of nerve injury that are likely influenced by ongoing spontaneous pain, which is difficult to measure but may be the most significant dependent measure worth investigating in preclinical studies of pain.

One guiding hypothesis of this work is that ongoing pain following nerve injury depresses mesolimbic reward circuitry, as well as its stimulation by opioids. This is expected to result in diminished responsiveness of nerve-injured rats to both the reinforcing effects of VTA ICSS, as well as its facilitation by opioids. Similarly, the other guiding hypothesis of this work is that nerve-injured rats develop ongoing spontaneous pain, and that manipulations (pharmacological or other) to the somatosensory system that alleviate ongoing spontaneous pain produce negative reinforcement that can be assessed using ICSS. Similarly, it is predicted that if ongoing pain following nerve injury depresses mesolimbic reward circuitry, that subsequent relief of ongoing pain will restore and/or stimulate this circuitry via disinhibition. Collectively, these studies attempt to provide key insights into how pain and reward systems interact; it is our hope that such information may be useful in the development of novel analgesic targets with low abuse liability.
References


Pierce, R. C., & Kumaresan, V. (2006). The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse?


CHAPTER II

OPIOID FACILITATION OF REWARDING ELECTRICAL BRAIN STIMULATION IS SUPPRESSED IN RATS WITH NEUROPATHIC PAIN

Eric E. Ewan and Thomas J. Martin

The following chapter is a manuscript published in Anesthesiology, volume 114, pages 624-632 (2011) and is reprinted with permission. Stylistic variations are due to conforming to the publishing journal. Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design and manuscript preparation.
Abstract

Introduction. Opioids are powerful analgesics but are also common drugs of abuse. Few studies have examined how neuropathic pain alters the pharmacology of opioids in modulating limbic pathways that underlie abuse liability.

Methods. Rats with or without spinal nerve ligation (SNL) were implanted with electrodes into the left ventral tegmental area and trained to lever press for electrical stimulation. The effects of morphine, heroin, and cocaine on facilitating electrical stimulation of the ventral tegmental area and mechanical allodynia were assessed in SNL and control subjects.

Results. Responding for electrical stimulation of the ventral tegmental area was similar in control and SNL rats. The frequency at which rats emitted 50% of maximal responding was 98.2 ± 5.1 Hz (mean ± s.e.m.) and 93.7 ± 2.8 Hz in control and SNL rats, respectively. Morphine reduced the frequency at which rats emitted 50% of maximal responding in control (maximal shift of 14.8 ±3.1 Hz) but not SNL (2.3 ± 2.2 Hz) rats. Heroin was less potent in SNL rats while cocaine produced similar shifts in control (42.3 ± 2.0 Hz) and SNL (37.5 ± 4.2 Hz) rats.

Conclusions. Nerve injury suppressed potentiation of electrical stimulation of the ventral tegmental area by opioids, suggesting that the positive reinforcing effects are diminished by chronic pain. Given concerns regarding prescription opioid abuse, developing strategies that assess both analgesia and abuse liability within the context of chronic pain may aid in determining which opioids are most suitable for treating chronic pain when abuse is a concern.
Introduction

Treatment of neuropathic pain with opioids remains controversial due to concerns regarding abuse potential. These concerns are highlighted by the fact that treatment of neuropathic pain typically requires much larger doses of opioids than those used to treat acute pain. Although much effort has been spent developing animal models to study the pathophysiology of this disease, few studies have addressed the extent to which neuropathic pain alters the rewarding effects of opioids which likely underlie opioid misuse in this population. This study addresses this issue by assessing opioid facilitation of rewarding electrical brain stimulation in rats with and without neuropathic pain.

Neuropathic pain suppresses the reinforcing effects of opioids in rodents. Dose response curves for mu opioid receptor (MOR) agonists in maintaining self-administration are shifted to the right in nerve-injured rats, and only doses that alleviate mechanical allodynia maintain self-administration following nerve injury. Similarly, nerve injury decreases morphine’s ability to induce conditioned place preference, a paradigm thought to be an indirect measure of a drug’s rewarding effects, in both rats and mice. A substantial literature implicates dopamine in mediating the rewarding effects of many abused drugs, including opioids. Specifically, dopaminergic neurons of the ventral tegmental area (VTA) project extensively to the nucleus accumbens, and release of dopamine in this region is thought to represent a key neural substrate for reward. The VTA contains MOR’s located predominantly on non-dopaminergic cells and opioids increase the firing of VTA dopaminergic neurons presumably via disinhibition of
dopaminergic cell bodies within the VTA. Previous work reveals that dopamine release in the nucleus accumbens following systemic administration of morphine is suppressed in nerve-injured rats. Collectively, these findings suggest that neuropathic pain alters classical reward circuitry in rodents, resulting in altered opioid pharmacology.

One particularly useful method to study opioid activity within the VTA is intracranial electrical self-stimulation (ICSS). ICSS is an operant paradigm that pairs an operant response with brief electrical stimulation of a discrete brain region. Rats will lever press to receive electrical stimulation of the VTA, which causes a substantial release of dopamine in the nucleus accumbens. Under these conditions, responding is directly related to the intensity and frequency of stimulation, such that intensity- and frequency-response curves can be generated that mirror pharmacological dose-response curves. Pharmacology studies reveal that dopamine and MOR agonists facilitate VTA ICSS, indicated by their ability to produce leftward shifts in VTA ICSS frequency response curves in rats. To this end, ICSS has proven to be a valuable tool for assessing the effects of drugs or environmental conditions on a discrete pathway within the limbic system, which is difficult to do with systemic drug self-administration. Therefore the application of this technique to a physiological or pharmacological question can complement drug self-administration studies.

The effect of spinal nerve ligation (SNL) on rewarding electrical brain stimulation and modulation by opioids has not been documented. Given that previous work suggests that morphine is less effective in stimulating dopamine
activity within the limbic system following nerve injury\textsuperscript{5}, we hypothesized that opioid facilitation of VTA ICSS would be suppressed in nerve-injured rats. We therefore assessed the ability of the MOR agonists morphine and heroin to shift VTA ICSS frequency-response curves to the left in rats with and without neuropathic pain. In addition, the anti-allodynic effects of each drug were assessed using von Frey filaments. Additionally, facilitation of VTA ICSS by cocaine was examined in SNL and control rats to determine if the effects of neuropathic pain were selective for opioids, or produced a generalized effect on this system.
Materials and Methods

Subjects

Subjects consisted of 26 male, Fisher 344 rats (9 SNL rats and 11 control rats were used exclusively for VTA ICSS, 4 SNL rats were used exclusively for determining the effects of drugs on paw withdrawal threshold (PWT), and 2 SNL rats were used for both VTA ICSS and determining drug effects on PWT). Rats weighed 300-350 g at the start of the experiment (Harlan Laboratories, Raleigh, NC), were group-housed, and were maintained on a reversed light-dark cycle (dark 05:00-17:00) in a temperature and humidity controlled environment immediately adjacent to the room in which all behavioral experiments were performed. Food and water were available ad libitum except during behavioral testing. All procedures were conducted in accordance with the guidelines adopted by the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Surgeries

*Electrode Implantation.* Rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal). Rats were placed in a stereotaxic frame and platinum bipolar stimulating electrodes (Plastics One, Roanoke, VA) were implanted into the left VTA at a 10° angle (2.3mm anterior to lambda, 0.6mm lateral from the midline, and 8.5mm below the skull surface). Electrodes were permanently secured to the skull by three
stainless steel screws embedded in dental acrylic. Rats were given penicillin G procaine (75,000 U, intramuscular) to prevent post-surgical infection.

**Spinal Nerve Ligation.** Immediately following electrode implantation, a portion of rats were subjected to SNL \(^{14}\). Briefly, a 3cm incision was made in the back using the iliac crests as a midpoint. An incision was then made in the underlying muscle, which was separated by both sharp and blunt dissection to expose the left transverse process of the fifth lumbar vertebra. The transverse process was removed using bone microrongeurs, and the fifth lumbar nerve was exteriorized from underneath the spinal column using a small metal hook and ligated using 4.0 silk suture with sufficient pressure to cause the nerve to bulge on each side of the ligature. The sixth lumbar nerve was exteriorized from underneath the iliac bone at the sciatic notch and ligated in a similar manner. All muscle layers were sutured using 4.0 chromic gut, the skin was sutured using 4.0 nylon suture, and exterior wounds were dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

**Paw Withdrawal Threshold**

To verify development of mechanical allodynia following SNL, PWT’s were determined according to previously published methods using von Frey filaments ranging in strength from 0.6 to 26.0 g (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL) \(^{15}\) for all animals using Dixon non-parametric statistics \(^{16}\). After a minimum of 14 days recovery from electrode implantation and SNL, withdrawal thresholds were determined and rats were considered alldynic if the withdrawal threshold was 4.0g or less; development of alldynia was a requisite for inclusion
of SNL rats in the current study. To determine if the drugs used during VTA ICSS alleviated established mechanical allodynia, baseline PWT’s were determined 20 minutes prior to intraperitoneal drug injections. PWT’s were then assessed 15, 60, and 120 minutes post-injection in a portion of rats subjected to SNL.

**Drugs**

Morphine sulfate was purchased as a 15 mg/ml sterile solution (Baxter Healthcare; Deerfield, IL). Heroin hydrochloride and cocaine hydrochloride were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD), dissolved in 0.9% (wt/vol) saline, and sterilized by filtration through a 0.22 μm nitrocellulose filter. All drugs were diluted using 0.9% (wt/vol) saline, pH 7.4.

**Intracranial Self-Stimulation (ICSS)**

*Apparatus.* Commercially available operant equipment was used consisting of an operant chamber containing a lever located 5 cm above a grid bar floor, a stimulus lamp located 2 cm above the lever, a house light located outside of the operant chamber, and a tone generator (Med Associates Inc., St. Albans, VT). The operant chamber was housed within a sound- and light- attenuating enclosure containing a ventilation fan. An ICSS stimulator controlled by a computer software program (Med Associates Inc.) that controlled all stimulation parameters and data collection was located outside of the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One) located above the operant chamber connected the electrodes to the ICSS stimulator via 25cm cables (Plastics One).
**Behavioral Procedure.** After a minimum of 14 days recovery from surgery, rats were trained to lever press for brain stimulation. Illumination of the stimulus light above the lever indicated stimulation availability. Each lever press resulted in a 0.5-sec train of rectangular alternating cathodal and anodal pulses (0.1-ms pulse durations), and was accompanied by the stimulus light turning off, the houselight illuminating, and operation of the tone. Responses during the 0.5-sec stimulation period provided no additional stimulation and were not recorded.

To determine the lowest stimulation intensity (current) that maintained high rates of responding, daily intensity-rate curves were generated. These 1-hr sessions consisted of six 10-minute components, with each component subdivided into ten 1-minute trials. Each trial began with a 5-sec timeout period, a 5-sec priming period in which rats received 5 noncontingent stimulations, and a 50-sec response period in which lever presses resulted in stimulation and were recorded. During these sessions the frequency of stimulation was held constant (156 Hz) and a series of 10 intensities (200-20 uA, 20uA increments corresponding to each trial) were presented in descending order. For data analysis, response rates during the first 2 components of each session were discarded since they were often highly variable and response rates during components 3-6 were averaged to create a single intensity-rate curve for each session. For each session the intensity that maintained 80% of maximal responding (EC80) was determined using Prism software (sigmoidal-dose response, variable slope; Graph Pad, La Jolla, CA), and responding was deemed stable when the EC80’s of three consecutive sessions varied by <10% of the
average EC80 of the three sessions. The average EC80 (intensity) of these three sessions was used for frequency-rate curve sessions, and was adjusted if needed.

Daily frequency-rate curves were generated similarly to the intensity-rate curves except that the intensity was held constant (unique to each animal) and a series of 10 frequencies (156-45 Hz, 0.06 log increments corresponding to each trial) were presented in descending order. Test sessions consisted of 7 components with a 15 or 60 minute timeout period between components 4 and 5, during which time rats received 1 mL/kg intraperitoneal injections of saline (0.9% wt/vol), morphine (0.3 – 6 mg/kg), heroin (0.03 – 1 mg/kg), or cocaine (0.3 – 10 mg/kg). For data analysis, the 2 components preceding drug injection (3 & 4) and the 3 components following drug injection (5, 6, & 7) were averaged and compared using Prism software (sigmoidal-dose response, variable slope; GraphPad). Drug test sessions were separated by at least 1 day. Saline was administered to each animal first, followed by administration of morphine or cocaine on alternate testing days. Heroin was tested after the morphine and cocaine dose-effect curves were completed followed finally by testing effective doses of morphine at the 60 min pretreatment time. Preliminary experiments were performed to determine the highest possible doses of each drug that could be administered without decreasing maximum response rates for VTA ICSS (data not shown in Results). All test sessions were performed between 1-5 months post surgery in both groups of rats. Additional animals were added to the
study as needed due to attrition from electrode loss or decreases in baseline responding for VTA ICSS with time.

**Histology**

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed and frozen in isopentane (-35°C) and were stored at -80°C. Coronal sections (25μm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the VTA (Figure 1).

**Data Analysis**

Data for PWT’s was analyzed using a two-way ANOVA with drug dose and time after infusion serving as the independent variables. The EF50 (frequency at which rats emitted 50% of maximal responding) and maximum response rate for VTA ICSS was calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad). The effect of drug treatment and SNL on VTA ICSS was analyzed using a two-way ANOVA with drug dose and treatment condition (SNL or control) serving as the independent variables and ΔEF50 (EF50 prior to injection – EF50 after injection) or maximal response rates serving as the dependent measures. Post-hoc analyses within the control or SNL groups were made using Dunnett’s t-test for multiple comparisons with saline injection serving as control. Post-hoc comparisons between control and SNL groups were made using Tukey’s HSD. A two-tailed p-value of 0.05 or less was considered statistically significant.
Figure 1. Schematic showing the location of stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats. Numbers next to diagrams indicate the distance anterior to the interaural line according to the atlas of Paxinos and Watson.\textsuperscript{24}
Results

Development of mechanical allodynia

PWT’s were significantly different between control and SNL rats implanted with VTA electrodes \[F(1,21)=71.0, \ p<0.0001\]. All rats implanted with VTA electrodes and subjected to SNL developed mechanical allodynia, with average PWT’s of \(2.21 \pm 0.25\) g (mean ± s.e.m., Figure 2). Rats implanted with VTA electrodes but not subjected to SNL did not develop mechanical allodynia, with average PWT’s of \(15.15 \pm 1.51\) (Figure 2).

Intracranial self-stimulation

Effect of SNL on baseline VTA ICSS. Electrical stimulation maintained responding in a frequency-dependent manner in both control and SNL rats. There was no significant effect of SNL on VTA ICSS compared to control rats with either the EF50 or the maximal response rate (Figure 2). The EF50 for VTA ICSS in SNL rats was \(93.7 \pm 2.8\) Hz and for control rats was \(98.3 \pm 5.1\) Hz \[F(1,20)=0.6, \ p=0.43\]. The maximal response rate in SNL rats was \(38.4 \pm 1.4\) resp/trial and for control rats was \(38.3 \pm 2.3\) resp/trial \[F(1,20)=0.001, \ p=0.98\].

Morphine. The effect of 15 min pretreatment with morphine in shifting the frequency response curves to the left was significantly different between control and SNL rats \[F(1,80)=6.1, \ p=0.02\], and this effect was dose-dependent \[F(4,80)=5.1, \ p=0.001\] (Figure 3). There was a significant interaction between SNL vs. control and morphine dose \[F(4,80)=2.7, \ p=0.03\]. In the control group morphine significantly increased ∆EF50 values at all doses of 1mg/kg and greater compared to saline \[F(4,41)=5.3, \ p=0.002\]. In contrast, in the SNL group
Figure 2. Development of mechanical allodynia following spinal nerve ligation (SNL) and baseline responding for electrical stimulation of the ventral tegmental area in control and SNL rats. (A) Paw withdrawal thresholds (PWT) were assessed between 14-21 days after surgery using von Frey filaments in control and SNL rats. (B) Frequency-response curves for baseline responding were generated by averaging the third and fourth components preceding saline administration (prior to any drug treatments). The y-axis indicates the number of self-stimulations (0.5-sec) during each 50-sec trial for each frequency (x-axis). Data shown are averages across control (n=11) and SNL (n=11) rats. Average current intensities during baseline responding were 139.6 (14.23) uA for control and 138.6 (9.83) uA for SNL rats. # Significantly different from control rats P \leq 0.05.
morphine did not significantly alter the \( \Delta \text{EF50} \) compared to saline at any dose \[F(4,38)=0.8, \ p=0.54\] and saline alone did not alter the \( \Delta \text{EF50} \) values in either group (p>0.05).

Morphine’s effect on ICSS in the VTA in control rats was greater after 1 hr compared to 15 min pretreatment; however morphine was still without effect in SNL rats (Figure 3). Analysis of the data with the longer morphine pretreatment time revealed a similar effect \[F(9,77)=6.4, \ p<0.0001\], with morphine’s effect being dose-responsive \[F(4,77)=7.3, \ p<0.0001\] and SNL producing a significant effect on morphine’s ability to shift the EF50 \[F(1,77)=9.4, \ p=0.003\]. As with the earlier time point, there was a significant interaction between SNL vs control and morphine dose \[F(4,77)=4.3, \ p=0.004\]. In control rats morphine produced significant leftward shifts in the frequency response curves, and \( \Delta \text{EF50} \) values were dose-dependent \[F(4,40)=8.9, \ p<0.001\] with all doses of 1 mg/kg and greater producing a significant effect. As with the earlier pretreatment time, morphine had no effect on the \( \Delta \text{EF50} \) values at any dose in SNL rats \[F(4,36)=1.0, \ p=0.4\] and saline alone did not alter the \( \Delta \text{EF50} \) values in either group following a 1 hr pretreatment (p>0.05).

Heroin. 15 min pretreatment with heroin altered ICSS in the VTA in both control and SNL rats, however significant differences were found between these two groups (Figure 4). Heroin shifted the frequency response curves to the left, producing significant increases in the \( \Delta \text{EF50} \) values in a dose-responsive manner in both control \[F(4,35)=6.5, \ p=0.006\] and SNL \[F(4,41)=8.1, \ p<0.0001\] rats. In the control group heroin significantly increased \( \Delta \text{EF50} \) values at all doses.
Figure 3. Effects of spinal nerve ligation (SNL) on morphine facilitation of electrical stimulation of the ventral tegmental area for drug incubation times of 15-min (A) and 60-min (B). Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (5-7) from the EF50 for the two components preceding drug injection (3-4) for each dose assessed. Data shown are averages across control (n=7-8) and SNL (n=6-7) rats. Frequency-response curves before and after 3mg/kg morphine (60-min) are shown for control (C, n=8) and SNL (D, n=7) rats. * Significantly different from saline treatment P ≤ 0.05. # Significantly different from SNL rats P ≤ 0.05.
Figure 4. Effects of spinal nerve ligation (SNL) on heroin facilitation of electrical stimulation of the ventral tegmental area. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (5-7) from the EF50 for the two components preceding drug injection (3-4) for each dose assessed. Data shown are averages across control (n=6-7) and SNL (n=7-8) rats. Frequency-response curves before and after 0.1 mg/kg heroin (15-min) are shown for control (B, n=6) and SNL (C, n=8) rats. * Significantly different from saline treatment P ≤ 0.05.
of 0.1mg/kg and greater compared to saline (p≤0.05). In the SNL group heroin significantly increased ∆EF50 values at all doses of 0.3mg/kg and greater compared to saline (p≤0.05).

Cocaine. 15 min pretreatment with cocaine altered ICSS in the VTA in both control and SNL rats in a similar manner (Figure 5). Cocaine shifted the frequency response curves to the left, producing significant increases in the ∆EF50 values in a dose-responsive manner in both groups of rats [control: F(4,36)=42.5, p<0.0001, SNL: F(4,41)=40.1, p≤0.0001]. Cocaine significantly increased ∆EF50 values at all doses of 1mg/kg and greater compared to saline (p≤0.05). There were no significant differences between control and SNL rats for any dose of cocaine.

Drug effects on maximal response rate. Both morphine and heroin had no effect on the maximum rate of responding maintained by intracranial stimulation in both groups at any time following injection. Cocaine had no effect on the maximum rate of responding in the control group [F(4,36)=1.0, p=0.4] but did slightly increase the maximum response rate in the SNL group [F(4,41=2.8, p=0.04] at the highest dose studied of 10 mg/kg (39.5±1.4 baseline, 43.5±2 treated, p<0.05).

Drug effects on mechanical allodynia
Morphine and heroin both increased PWT over the range of doses that produced significant increases in ∆EF50 for ICSS (Figure 6). The effects of morphine were dose-dependent [F(3,23)=15.6, p<0.0001; F(3,23)=27.2, p<0.0001, 15 or 60 min post-injection, respectively]. Both 3 and 6 mg/kg morphine significantly increased
Figure 5. Effects of spinal nerve ligation (SNL) on cocaine facilitation of electrical stimulation of the ventral tegmental area. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (5-7) from the EF50 for the two components preceding drug injection (3-4) for each dose assessed. Data shown are averages across control (n=6-7) and SNL (n=7-8) rats. Frequency-response curves before and after 10 mg/kg heroin (15-min) are shown for control (B, n=7) and SNL (C, n=8) rats. * Significantly different from saline treatment P ≤ 0.05.
A. 15-min Incubation

Reduction in EF 50 (Hz)

Cocaine (mg/kg)

B. Control - 10mg/kg Cocaine

Responses

Frequency (Hz)

C. SNL - 10mg/kg Cocaine

Responses

Frequency (Hz)
Figure 6. Drug effects on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. Dose-effect curves were determined for morphine (A), heroin (B), and cocaine (C) on PWT in SNL rats at the indicated times following an intraperitoneal injection (n=6). * Significantly different from saline treatment P ≤ 0.05.
A  Morphine

- Saline
- 1 mg/kg
- 3 mg/kg
- 6 mg/kg

B  Heroin

- Saline
- 0.1 mg/kg
- 0.3 mg/kg
- 1 mg/kg

C  Cocaine

- Saline
- 5 mg/kg
- 10 mg/kg
PWT at these time points compared to saline injection (p<0.05), and the maximum effect occurred 60 min following injection of 6 mg/kg morphine, resulting in a PWT of 16.6±2.1 g. Heroin also produced dose-dependent increases in PWT [F(3,23)=38.4, p<0.0001; F(3,23)=22.8, p<0.0001, 15 or 60 min post-injection, respectively]. Both 0.3 and 1.0 mg/kg of heroin significantly increased PWT at these time points compared to saline (p<0.05), and the maximum effect of heroin (22.1±0.8 g) was greater than that of morphine, occurring 15 min after injection of 1 mg/kg (p≤0.05). Cocaine had no effect on PWT at any dose [F(2,17)=1.6, p=0.2; F(2,17)=1.1, p=0.4, 15 or 60 min post-injection, respectively] (Figure 6). Saline had no effect on PWT at any time following injection [F(2,17)=0.2, p=0.8] (Figure 6).
Discussion

Given the widespread concerns regarding opioid misuse in chronic pain patients, there is a need to determine to what extent the presence of chronic pain alters the abuse potential of opioids. The current study indicates that MOR agonists become less effective in facilitating VTA ICSS following peripheral nerve injury, suggesting that their ability to produce positive reinforcement is diminished by the presence of chronic pain. The diminished effects in SNL rats appear to be specific to opioids, since cocaine was equally effective in facilitating VTA ICSS in both control and SNL rats. These data support our hypothesis that the efficacy of opioids in stimulating the mesolimbic dopaminergic system is suppressed in rats with neuropathic pain.

It is important to highlight differences between the effects of SNL on suppressing morphine and heroin facilitation of ICSS. The facilitating effects of morphine were suppressed to such an extent following SNL that no dose of morphine (0.3 – 6 mg/kg) produced leftward shifts in the frequency response curves following SNL. The complete suppression of morphine’s facilitating effects in SNL rats following 15-min drug incubation was surprising; to further confirm these findings a 60-min pretreatment was used which produced even greater leftward shifts in the frequency rate curves in control subjects, and again morphine failed to facilitate ICSS selectively in SNL rats. In contrast, only one dose of heroin assessed (0.1mg/kg) produced a significant leftward shift in control rats that was not observed in SNL rats; meanwhile heroin was still effective in facilitating VTA ICSS at the two highest doses assessed (0.3 &
1mg/kg) in control and SNL rats. Doses higher than 6mg/kg morphine and 1mg/kg heroin decreased maximal response rates for ICSS (data not shown), and therefore the dose range was restricted to 6mg/kg and 1mg/kg, respectively. From these data, one would predict that morphine and heroin would produce positive reinforcement in control rats, but that only heroin would produce positive reinforcement in SNL rats, albeit at larger doses. Interestingly, this prediction is supported by previous work that assessed opioid self-administration in rats with and without SNL \(^4\). In those experiments, SNL decreased maximal response rates for morphine self-administration compared to SHAM rats to such an extent that responding for morphine in SNL rats no longer was dose-dependent; morphine essentially appeared to not serve as a reinforcer following SNL. In contrast, heroin self-administration was merely shifted to the right in SNL rats compared to SHAM rats, and maximal rates of responding were unaffected; heroin still served as a reinforcer at higher doses following SNL. Taken together, suppression of the reinforcing effects of opioids in each of these paradigms seems to be related to the efficacy of the opioid, such that the reinforcing effects of lower efficacy opioids (morphine) are completely diminished following SNL whereas those of higher efficacy opioids (heroin) can be overcome by increasing the dose.

The mechanism underlying the loss of opioid facilitation of VTA ICSS following SNL appears to not be general disruption of limbic activity. The ability of electrical stimulation of the VTA to maintain operant responding is not altered by SNL; the frequency-response curves and the mean intensities required to maintain
responding do not differ between SNL and control rats. Additionally, cocaine’s facilitating effects on VTA ICSS were the same in SNL and control rats suggesting that SNL does not cause a nonspecific disruption in behavior or limbic activity. Therefore the effects appear to be unique to opioids, leaving the possibility that SNL produces a selective disruption in MOR activity. In particular, alterations in MOR G-protein signaling may occur in the VTA, leading to reduced signaling following MOR agonist activation and ultimately suppression of opioid facilitation of VTA ICSS. Previous work supports this notion, since it was shown that morphine-stimulated GTPγS binding in the VTA and morphine-induced dopamine release in the nucleus accumbens are suppressed in nerve-injured rats. Similar effects were observed in nerve-injured mice, however the mechanism for receptor uncoupling within this circuitry is unclear. It is also unclear if such uncoupling can be reversed with acute or chronic blockade of pain transmission with analgesics, or if the alterations are irreversible.

MOR signaling may be altered following SNL in other brain regions that influence activity within the VTA or receive output from the VTA. Other brain regions that would most likely be involved are those receiving dopaminergic output from the VTA, including the nucleus accumbens, ventral pallidum, and amygdala, all of which contain MOR's. To address this, future studies could examine the degree to which chronic pain alters the facilitating effects of opioids administered locally into each of these discrete brain regions. Given the knowledge of which MOR's are and are not responsible for this effect, the
underlying neural mechanisms responsible for loss of opioid efficacy in facilitating VTA ICSS could be explored.

The suppression of opioid facilitation of rewarding brain stimulation following nerve injury has important implications regarding opioid abuse potential in the presence of chronic pain. Most drugs of abuse, including opioids, increase dopamine transmission throughout the mesolimbic dopaminergic system, and their ability to facilitate VTA ICSS is often used as a measure of their abuse potential. Therefore, a reduction in the facilitating effects of opioids on VTA ICSS following SNL indicates a loss of the positive rewarding effects that likely contribute to opioid abuse. Clinically, the notion that opioid abuse potential is reduced by the presence of chronic pain is not new. A recent review suggests that abuse/addiction rates in chronic pain patients are relatively low (−3%), although precise rates for opioid misuse remain difficult to determine.

Since this is the first report of opioid effects on VTA ICSS during chronic pain, it is unclear if similar suppression of VTA ICSS will be observed with other opioid compounds. Additionally, it is unclear to what extent drug history alters the facilitatory effects of opioids on VTA ICSS. In the current study rats received all drug conditions, and although repeated testing could potentially impact facilitation of ICSS, the fact that drug history was similar between control and SNL rats suggests that these effects are likely negligible. Additionally, morphine injections were given at the beginning of the study at the 15 min time point and at the end of the study at the 60 min pretreatment condition. Similar potencies were found for facilitation of VTA ICSS in the control group, and morphine had no effect on
VTA ICSS in the SNL group, suggesting that drug history likely had a minor role if any on these data. An interesting question with respect to drug history would be if extensive drug exposure (months) prior to nerve-injury would alter the abuse liability of opioids as measured using VTA ICSS. This question is particularly interesting given that one of the greatest predictors of opioid abuse/addiction in chronic pain patients is a previous history of drug abuse\textsuperscript{18}. Ultimately, VTA ICSS has great potential as a preclinical screening tool for assessing the abuse liability of potential opioid as well as nonopioid analgesics during chronic pain.

It was surprising that no differences were observed in baseline responding for VTA ICSS between control and SNL rats. Administration of lactic acid into the peritoneal cavity produces abdominal pain and irritation and has recently been shown to suppress responding for ICSS, an effect that is reversed by pretreatment with morphine\textsuperscript{19}. These data suggest that the presence of severe acute pain diminishes the reinforcing effects of electrical stimulation of ascending dopamine pathways. Lactic acid injected into the peritoneal cavity produces overt abdominal writhing and stretching behavior, and rats will typically cease ongoing behavior during these episodes, which are inhibited by a variety of analgesics including opioids.\textsuperscript{19} SNL produces few overt behavioral effects however. The difference between the effects of intraperitoneal lactic acid and SNL on ICSS may be the presence of these pronounced behavioral effects, the relative severity of the pain stimulus, or differential effects on the mesolimbic reward pathways. Given that people with chronic pain often suffer from affective disorders such as anxiety and depression\textsuperscript{20}, which suggests that persistent pain
leads to an overall negative affective state, it was expected that nerve injury would reduce activity of reward pathways originating within the limbic system, and therefore that SNL rats would be less responsive to rewarding electrical stimulation. However the current data suggest that if SNL decreases activity within the limbic system in rats, that VTA ICSS lacks the sensitivity to detect such an effect of this manipulation. Given that the central amygdala (CeA) is activated during pain states 21,22 and sends GABAergic projections that synapse directly on dopaminergic cell bodies of the VTA 23, it is plausible that chronic pain increases the activity of these CeA GABAergic neurons, causing tonic suppression of dopamine transmission from the VTA in neuropathic rats. The present data suggest however that the basal activity of reward pathways are not significantly inhibited following SNL, at least not to an extent that can be detected using the ICSS methodology. Instead, the present data suggest that dopaminergic reward pathways originating from the VTA are altered in a highly selective manner following SNL in rats, resulting in a specific alteration in opioid pharmacology within this circuitry.

In conclusion, the ability of opioids to facilitate VTA ICSS is suppressed following SNL. This suggests that opioids are less effective in stimulating dopamine transmission originating in the VTA. The effect appears to be restricted to opioids since facilitation of VTA ICSS with cocaine is maintained in neuropathic rats. The current methodology used (VTA ICSS) not only complements previous work using drug self-administration, but adds an additional layer of precision by isolating opioid effects within limbic reward
circuitry, which is difficult to do using systemic drug self-administration. Future work should focus on whether similar effects occur with different opioid compounds, as well as determine the underlying mechanisms responsible for opioid suppression following nerve injury. The use of operant techniques such as VTA ICSS may be beneficial as a preclinical tool for screening the abuse liability of novel analgesics, as well as provide useful information regarding the interactions between chronic pain and classical reward circuitry.
Acknowledgements

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland. The authors wish to thank Dr. Steve Negus (PhD, Professor, Dept. of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA, USA) for his helpful advice and discussion regarding the intracranial self-stimulation procedure and data analysis.
References


CHAPTER III

REWARDING ELECTRICAL BRAIN STIMULATION IN RATS FOLLOWING PERIPHERAL NERVE INJURY: DECREASED FACILITATION BY COMMONLY ABUSED PRESCRIPTION OPIOIDS.

Eric E. Ewan and Thomas J. Martin

The following chapter is accepted pending revision in *Anesthesiology*, and is reprinted with permission. Stylistic variations are due to conforming to the publishing journal. Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design and manuscript preparation.
Abstract

**Introduction.** Prescription opioid abuse is a significant concern in treating chronic pain, yet few studies examine how neuropathic pain alters the abuse liability of commonly abused prescription opioids.

**Methods.** Normal and spinal nerve ligated (SNL) rats were implanted with electrodes into the left ventral tegmental area (VTA). Rats were trained to lever press for intracranial electrical stimulation (VTA ICSS), and the effects of methadone, fentanyl, hydromorphone, and oxycodone on facilitation of VTA ICSS were assessed. A second group of neuropathic rats were implanted with intrathecal catheters and the effects of intrathecal clonidine, adenosine, and gabapentin on facilitation of VTA ICSS were assessed. The effects of electrical stimulation of the VTA on mechanical allodynia were assessed in SNL rats.

**Results.** Responding for VTA ICSS was similar in control and SNL rats. Methadone, fentanyl, and hydromorphone were less potent in facilitating VTA ICSS in SNL rats. Oxycodone produced a significant facilitation of VTA ICSS in control (maximum shift 24.10 ± 6.19 Hz) but not SNL rats (maximum shift 16.32 ± 7.49 Hz), but also reduced maximal response rates in SNL rats. Intrathecal administration of clonidine, adenosine, and gabapentin failed to facilitate VTA ICSS in SNL rats, and electrical stimulation of the VTA did not alter mechanical allodynia following nerve injury.

**Conclusions.** The present data suggests that the positive reinforcing effects of commonly abused prescription opioids are diminished following nerve injury. Additionally, alleviation of mechanical allodynia with non opioid analgesics does
not appear to stimulate limbic dopamine pathways originating from the VTA in SNL rats.
Introduction

Significant issues regarding the treatment of chronic pain with opioids remain due to concerns over misuse and diversion of medications to illicit drug markets.\textsuperscript{1,2} Prescription opioid abuse is now the fastest growing drug abuse problem in the United States,\textsuperscript{3} and presents a significant economic burden.\textsuperscript{4} A recent study of patients presenting for treatment of dependence on prescription opioids reports that the most commonly abused narcotics include oxycodone (79%), hydrocodone (67%), methadone (40%), morphine (29%), hydromorphone (16%), and fentanyl (9%).\textsuperscript{5} Few preclinical studies exist, however, that examine the abuse potential of these drugs in the context of pain.

Laboratory investigations aimed at understanding the interaction between pain and addiction have begun to provide some evidence that chronic pain suppresses opioid reward.\textsuperscript{6} Spinal nerve ligated (SNL) rats require larger doses of opioids to maintain intravenous self-administration.\textsuperscript{7} Nerve injury also decreases morphine induced conditioned place preference (CPP) in rats\textsuperscript{8} and mice.\textsuperscript{9} Similarly, direct injection of the \(\mu\) opioid receptor agonist DAMGO into the ventral tegmental area (VTA), an area implicated in opioid reward, produces CPP that is suppressed in nerve-injured mice.\textsuperscript{10}

Behavioral paradigms such as self-administration and CPP have been used extensively for assessment of drug reward, yet in the context of chronic pain, assessment of reward may be complicated by a drug’s analgesic effects. For instance, self-administration of opioids in SNL rats occurs only at doses that also alleviate mechanical allodynia, and administration of the spinal analgesic
clonidine reduces opioid intake selectively in nerve-injured rats.\textsuperscript{7} Moreover, spinal clonidine alone produces CPP\textsuperscript{11} and maintains intrathecal self-administration selectively in nerve-injured rats at doses that alleviate mechanical allodynia.\textsuperscript{12} One explanation of these data is that alleviation of mechanical allodynia serves as a direct reinforcing stimulus in rats following nerve injury. Another possible explanation, however, is that alleviation of mechanical allodynia indirectly stimulates classic reward pathways thought to be primarily responsible for the abuse potential of drugs.

Intracranial self-stimulation (ICSS) is a technique that has been used to evaluate the rewarding effects of drugs. Rats can be trained to lever press for electrical stimulation of the VTA, which is directly influenced by the intensity and frequency of stimulation.\textsuperscript{13} VTA stimulation increases dopamine neurotransmission in the nucleus accumbens.\textsuperscript{14} The VTA is implicated in opioid reward; opioids increase the firing of VTA dopaminergic neurons via disinhibition,\textsuperscript{15,16} and subsequently reduce the intensity and/or frequency of stimulation required to maintain responding for VTA ICSS in rats (facilitation).\textsuperscript{17} Recently we reported that morphine and heroin facilitation of VTA ICSS is suppressed in SNL rats.\textsuperscript{18}

Given the growing prescription opioid abuse problem, one goal of the current work was to examine the effects of nerve injury on the rewarding effects of commonly abused prescription opioids using VTA ICSS. Given that spinal clonidine elicits rewarding effects in animals with neuropathic pain,\textsuperscript{11,12} a second goal of the current work was to determine if alleviation of mechanical allodynia
with analgesics stimulates the mesolimbic dopamine system using VTA ICSS in SNL rats.
Materials and Methods

Subjects

Subjects consisted of 42 male, Fisher 344 rats (8 SNL and 9 control rats for assessment of opioids on VTA ICSS; 7 SNL rats for assessment of opioids on paw withdrawal threshold (PWT); 4 SNL rats for assessment of spinal analgesics on VTA ICSS and PWT, 4 SNL rats for assessment of morphine on VTA ICSS, and 3 SNL rats for both spinal analgesics and morphine; 7 control rats for assessment of morphine on VTA ICSS). Rats weighed between 300-350 g at the beginning of the experiment (Harlan Laboratories, Raleigh, NC). All rats were group-housed, except for those receiving intrathecal catheters, and were maintained on a reversed light-dark cycle (dark 05:00-17:00). Rats were housed in a temperature and humidity controlled room that was adjacent to the room where behavioral experiments were performed. Food and water were available ad libitum with the only exception being during behavioral experiments. All procedures were conducted according to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Surgeries

Electrode Implantation. Rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10 mg/kg, intraperitoneal), and received penicillin G procaine (75,000 U, intramuscular) as a preventative measure against infection. After being placed in a stereotaxic frame, platinum
bipolar stimulating electrodes (Plastics One, Roanoke, VA) were implanted into the left VTA at a 10° angle (2.3mm anterior to lambda, 0.6mm lateral from the midline, and 8.5mm below the skull surface). Three stainless steel screws embedded in dental acrylic permanently secured each electrode to the skull surface.

Spinal Nerve Ligation. Immediately following electrode implantation, 26 of the 42 rats were subjected to SNL as previously described. Briefly, a 3cm incision was made through the skin and underlying muscle of the lower back, which was separated, and the left transverse process of the fifth lumbar vertebra was removed using bone microrongeurs. The fifth lumbar nerve was then exteriorized and ligated using 4.0 silk suture. The sixth lumbar nerve was exteriorized from below the iliac bone at the sciatic notch and similarly ligated. Each ligation caused the nerve to bulge on each side of the ligature. Muscle layers were sutured with 4.0 chromic gut, the skin with 4.0 nylon suture, and exterior wounds dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

Intrathecal Catheter Implantation. Rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal), and received penicillin G procaine (75,000 U, intramuscular) as a preventative measure against infection. Rats were then implanted with intrathecal catheters according to previously described methods. Briefly, rats were placed in a stereotaxic frame with the head bent downward. An incision was made through the skin and muscle of the neck, which was retracted, and a small hole was made in the atlanto-occipital membrane through which an 8.5 cm catheter was
inserted. The spinal catheter was constructed from 32 g polyethylene tubing (ReCathCo, Allison Park, PA) fused to biocompatible Tygon tubing (inner diameter 0.01”, formulation S-54-HL, Saint-Gobain Plastics Inc., Akron, OH) using cyclohexanone. The catheter was secured to the surrounding muscle with 5.0 Vicryl suture (Ethicon Inc., Cornelia, GA). The skin was sutured with 4.0 nylon suture and exterior wounds dressed with antibiotic powder.

**Paw Withdrawal Threshold**

Mechanical allodynia following SNL was assessed by measuring PWT’s using von Frey filaments (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL)\(^{19}\) for all animals using Dixon non-parametric statistics.\(^{20}\) Following at least 14 days post-surgery, PWT’s were assessed; allodynia was defined as a PWT of less than 4.0g. During assessment of drug effects on mechanical allodynia, baseline PWT’s were determined 20 minutes prior to intraperitoneal or intrathecal drug injections. PWT’s were then assessed 15, 60, and 120 minutes following intraperitoneal injection, and 60 minutes following intrathecal injection in SNL rats. To determine if VTA stimulation alleviated established mechanical allodynia, noncontingent electrical stimulation was delivered using similar parameters to those during self-stimulation sessions. During stimulation testing special care was taken to prevent the occurrence of motor abnormalities that would otherwise interfere with behavioral testing. First, baseline PWT’s were assessed immediately prior to stimulation. Next, the frequency of stimulation was set to 45 Hz and the current to 10uA; the current was then gradually increased (10uA increments) to that which was used during self-stimulation sessions (unique to
each rat). Finally, PWT’s were assessed in ascending order (45, 68, 103, 136, 156Hz), with the frequency of stimulation being gradually increased (10Hz increments until the next scheduled test frequency) after each PWT assessment. The entire testing procedure lasted between 6-8 minutes and successfully prevented the development of motor abnormalities.

Drugs

Methadone hydrochloride, fentanyl hydrochloride, and oxycodone hydrochloride were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD), dissolved in 0.9% (wt/vol) saline, and sterilized by filtration through a 0.22 μm nitrocellulose filter. Hydromorphone hydrochloride was purchased as a 2 mg/ml sterile solution (Hospira Inc., Lake Forest, IL). Morphine sulfate was purchased as a 15 mg/ml sterile solution (Baxter Healthcare; Deerfield, IL). Clonidine hydrochloride was purchased from Sigma-Aldrich Co. (St. Louis, MO). Adenosine was purchased as a 3mg/ml sterile solution (Fujisawa USA Inc., Deerfield, IL). Gabapentin was purchased from Tocris Bioscience (Ellisville, MO). All drugs were diluted using 0.9% (wt/vol) saline, pH 7.4.

Intracranial Self-Stimulation (ICSS)

**Apparatus.** An operant chamber with a lever 5 cm above a grid bar floor, stimulus lamp 2 cm above the lever, and tone generator was used (Med Associates Inc., St. Albans, VT). The operant chamber was housed within a sound- and light-attenuating enclosure containing a houselight and ventilation fan. An ICSS stimulator controlled by a computer software program (Med
Associates Inc.) that controlled all stimulation parameters and data collection was located outside of the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One) located above the operant chamber connected the electrodes to the ICSS stimulator via 25cm cables (Plastics One).

**Behavioral Procedure.** Following at least 14 days post-surgery, rats were trained to lever press for brain stimulation as previously described\(^{18}\). A stimulus light located above the lever indicated stimulus availability. Lever presses produced a 0.5-sec train of rectangular alternating cathodal and anodal pulses (0.1-ms pulse durations); during stimulation the stimulus light turned off, the houselight turned on, and a tone was sounded. Responding during the 0.5-sec stimulation period resulted in no further stimulation and was not recorded.

During initial acquisition sessions the frequency was held constant (156Hz) and the intensity was adjusted by the experimenter to determine the lowest intensity that maintained high rates of responding (>40 responses/minute). Once responding was established frequency-rate curves were generated. These 2-hr sessions consisted of six 10-min components, which were further broken down into ten 1-min trials. Each 60-sec trial consisted of a 5-sec timeout, then a 5-sec period during which five noncontingent stimulations were delivered, and finally a 50-sec period in which lever presses resulted in stimulation and were recorded. During these sessions the intensity remained the same (unique to each animal) and 10 frequencies (156-45 Hz, 0.06 log increments) corresponding to each trial were presented in descending order. A 1-hr timeout period between components 3 and 4 permitted drug injections during test sessions. During test sessions when
fentanyl was administered, the timeout period was reduced to 15-min. At the beginning of the timeout period rats received 1 mL/kg intraperitoneal injections of saline (0.9% wt/vol), morphine (3mg/kg), methadone (0.3 – 6 mg/kg), fentanyl (0.01 – 0.1 mg/kg), hydromorphone (0.03 – 1 mg/kg), or oxycodone (0.3 – 3 mg/kg); when testing intrathecal drugs rats received 20uL (5uL drug followed by 15uL saline flush) intrathecal injections of saline (0.9% wt/vol), clonidine (3 & 10ug), adenosine (30ug) or gabapentin (100ug). For data analysis, the 2 components preceding drug injection (2 & 3) and the 3 components following drug injection (4, 5, & 6) were averaged and compared using Prism software (sigmoidal-dose response, variable slope; Graph Pad, La Jolla, CA). All test sessions were separated by at least 1 day. Saline was administered to each animal first, followed by subsequent administrations of methadone, fentanyl, hydromorphone, and oxycodone; for spinal analgesic testing rats were initially tested with intraperitoneal morphine (3mg/kg) first, followed by intrathecal administration of clonidine, adenosine, and gabapentin. Preliminary experiments determined the highest doses of each opioid that could be delivered without decreasing maximum response rates for VTA ICSS (data not shown in Results). All test sessions were performed between 1-4 months after surgery, and animals were added when needed due to attrition from electrode loss or decreased responding for VTA ICSS over time.

**Histology**

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed and frozen in isopentane (-35°C) and were stored at -80°C. Coronal
sections (25μm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the VTA (Figure 1).

**Data Analysis**

Data for PWT’s was analyzed using a two-way ANOVA with drug dose and time after infusion serving as the independent variables. The EF50 (frequency at which rats emitted 50% of maximal responding) and maximum response rate for VTA ICSS was calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad, San Diego, CA). The effect of drug treatment on VTA ICSS was analyzed using a one-way ANOVA with drug dose serving as the independent variables and ΔEF50 (EF50 prior to injection – EF50 after injection) or maximal response rates serving as the dependent measures. Post-hoc analyses within the control or SNL groups were made using Dunnett’s t-test for multiple comparisons with saline injection serving as control. A two-tailed p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc., Cary, NC).
Figure 1. Location of the stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats. Numbers left of each brain section indicate distance anterior to the interaural line, according to the atlas of Paxinos and Watson.²⁸
Results

Effects of SNL and morphine on VTA ICSS.

Consistent with previous results,\textsuperscript{18} the EF50 and Rmax for VTA ICSS was similar for control and SNL rats (figure 2A). The EF50 for VTA ICSS in control rats was 100.2 (2.7) Hz (mean±SEM) and for SNL rats was 100.2 (2.7) Hz [F(1,16)=0, p=1.0]. The Rmax was 43.2 (2.3) and 39.9 (2.4) responses/component for control and SNL rats, respectively [F(1,16)=1.0, p=0.3]. The stimulus intensities used for ICSS also did not differ between control (168 ± 20 µA) and SNL (144 ± 22 µA) rats [F(1,16)=0.6, p=0.4].

Also consistent with previous data,\textsuperscript{18} morphine (3 mg/kg, intraperitoneal) in control rats produced a leftward shift in the frequency rate curve for VTA ICSS, reducing the EF50 from 93.3 (2.4) Hz [mean(SEM)] to 84.2 (3.4) Hz [F(1,13)=4.8, p=0.048] (Figure 2B). Morphine had no effect on VTA ICSS in SNL rats however as shown previously [F(1,13)=0.8, p=0.4] (Figure 2C).\textsuperscript{18} Saline administration had no effect on the EF50 for VTA ICSS in either control [F(1,13)=0.2, p=0.6] or SNL [F(1,13)=0.0004, p=0.98] rats. Neither saline nor morphine altered Rmax for VTA ICSS in either the control [F(1,13)=0.0007, p=0.98; F(1,13)=0.08, p=0.8, respectively] or SNL [F(1,13)=1.5, p=0.2; F(1,13)=0.01, p=0.9, respectively] rats.

Effects of opioids on VTA ICSS in control and SNL rats.

Fentanyl. Fentanyl shifted the frequency-rate curves to the left in both control and SNL rats. The effect of fentanyl on $\Delta$EF50 in control rats was dose-dependent [F(4,36)=15.6, p<0.0001] with all doses greater than 0.01 mg/kg producing a significant leftward shift in the frequency rate curve (p≤0.05) (Figure
Figure 2. Baseline responding for electrical stimulation of the ventral tegmental area (VTA ICSS) and the effects of 3mg/kg morphine on facilitation of VTA ICSS in control and spinal nerve ligated (SNL) rats. (A) Frequency-response curves for baseline responding were generated by averaging the second and third components preceding saline administration (prior to any drug treatments). The y-axis indicates the number of self-stimulations (0.5-sec) during each 50-sec trial for each frequency (x-axis). Data shown are averages across control (n=9) and SNL (n=8) rats. Average current intensities during baseline responding were 168.33 (21.75) uA for control and 144.38 (20.03) uA for SNL rats. Frequency-response curves before and after 3mg/kg morphine (60-min) are shown for control (B, n=7) and SNL (C, n=7) rats.
The effect of fentanyl on $\Delta$EF50 was also dose-dependent in SNL rats $[F(4,35)=8.0, p=0.001]$ with only the two highest doses given (0.06 and 0.1 mg/kg) producing a significant shift in the frequency-rate curve compared to saline ($p\leq0.05$) (Figure 3). Fentanyl did not alter the Rmax at any dose in either control $[F(4,36)=0.7, p=0.6]$ or SNL $[F(4,35)=1.8, p=0.2]$ rats.

**Methadone.** Methadone significantly shifted the frequency rate curves for VTA ICSS to the left, in a manner similar to fentanyl, in both control and SNL rats. The effect of methadone on $\Delta$EF50 was dose-dependent in control rats $[F(4,36)=4.7, p=0.004]$, with 3.0 and 6.0 mg/kg resulting in a $\Delta$EF50 significantly different from saline treatment ($p\leq0.05$) (Figure 4A). The effect of methadone on $\Delta$EF50 was also dose-dependent $[F(4,39)=4.5, p=0.005]$, with only the 6.0 mg/kg dose producing a $\Delta$EF50 significantly different from saline in SNL rats ($p\leq0.05$) (Figure 4A). As with fentanyl, methadone did not alter the Rmax in either control or SNL rats [control: $F(4,36)=1.4, p=0.2$; SNL: $F(4,39)=1.1, p=0.4$].

**Hydromorphone.** Hydromorphone potentiated VTA ICSS in a manner similar to that of methadone and fentanyl, shifting the frequency-rate curves to the left without altering the maximum rate of responding, in both control and SNL rats. The effect of hydromorphone on $\Delta$EF50 was dose-dependent in control rats $[F(4,36)=8.2, p=0.0001]$ with doses of 0.3 and 1.0 mg/kg producing a significantly greater effect than saline ($p\leq0.05$) (Figure 4B). Hydromorphone increased $\Delta$EF50 values in a dose-dependent manner in SNL rats as well $[F(4,35)=5.6, p=0.002]$, with only the highest dose of 1.0 mg/kg producing an effect significantly different from that produced by saline ($p\leq0.05$) (Figure 4B). Hydromorphone did not alter
Figure 3. Effects of spinal nerve ligation (SNL) on fentanyl facilitation of electrical stimulation of the ventral tegmental area. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (4-6) from the EF50 for the two components preceding drug injection (3-4) for each dose assessed. Data shown are averages across control (n=7) and SNL (n=7) rats. Frequency-response curves before and after 0.03mg/kg fentanyl (15-min) are shown for control (B, n=7) and SNL (C, n=7) rats. * Significantly different from saline treatment P ≤ 0.05.
Figure 4. Effects of spinal nerve ligation (SNL) on methadone (A), hydromorphone (B), and oxycodone (C) facilitation of electrical stimulation of the ventral tegmental area. Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (4-6) from the EF50 for the two components preceding drug injection (2-3) for each dose assessed. Data shown are averages across control (n=7 for methadone and hydromorphone; n=6 for oxycodone) and SNL (n=8 for methadone; n=7 for hydromorphone and oxycodone, with the exception of n=6 for 3mg/kg Oxycodone) rats. * Significantly different from saline treatment P ≤ 0.05.
Rmax for VTA ICSS in either control or SNL rats [saline: F(4,36)=0.3, p=0.9; SNL: F(4,35)=0.2, p=0.9].

**Oxycodone.** Oxycodone shifted the frequency-rate curve for VTA ICSS in control rats in a dose-dependent manner [F(4,32)=7.0, p=0.0005] with doses of 1 and 3 mg/kg increasing ΔEF50 greater than saline administration (p≤0.05) (Figure 4C). Oxycodone’s effect on ΔEF50 in SNL rats was not dose-dependent however [F(4,34)=2.1, p=0.1] (Figure 4C). As with the other opioids tested oxycodone did not alter Rmax in control rats [F(4,32)=0.3, p=0.8], but did decrease Rmax in SNL rats [F(4,34)=3.0, p=0.04], with Rmax being significantly lower following administration of 1 mg/kg compared to saline (p≤0.05).

**Effects of opioids on mechanical allodynia in SNL rats.**

All of the opioids administered produced a reversal of mechanical allodynia in SNL rats in the range of doses that were examined using VTA ICSS (Figure 5). Administration of saline (1 ml/kg intraperitoneal) did not alter PWT at any time point [F(3,27)=0.5, p=0.7]. Methadone produced a time- and dose-dependent anti-allodynic effect [time: F(3.111)=11.2, p<0.0001; dose: F(3,111)=15.1, p<0.0001] and there was a significant interaction between time and dose [F(9,111)=3.2, p=0.002]. Only the highest dose of methadone (6 mg/kg) produced a significant effect on PWT compared to saline (p≤0.05). Fentanyl also produced an anti-allodynic effect that was dependent upon dose [3,111)=9.9, p<0.0001] and time after administration [F(3,111)=15.9, p<0.0001], with a significant interaction between time and dose [F(9,111)=3.9, p=0.0003]. The maximum effect of fentanyl occurred 15 min after administration and both 0.06
Figure 5. Opioid effects on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. Dose-effect curves were determined for opioids on PWT in SNL rats. Data shown are average PWT’s 15-min (fentanyl) or 60-min (methadone, hydromorphone, and oxycodone) following intraperitoneal injection (n=7). * Significantly different from saline treatment P ≤ 0.05.
and 0.1 mg/kg produced an effect significantly greater than saline (p≤0.05). Hydromorphone reversed mechanical allodynia in a time- [F(3,111)=8.7, p<0.0001] and dose- [F(3,111)=9.0, p<0.0001] dependent manner with a significant interaction between time and dose [F(9,111)=2.9, p=0.005]. The maximum effect of hydromorphone was found 1 hr after administration, with only the highest dose of 1.0 mg/kg producing an effect significantly greater than that of saline (p≤0.05). The effects of oxycodone were dependent upon dose [F(3,111)=31.1, p<0.0001] and time after administration [F(3,111)=23.5, p<0.0001] and there was a significant interaction between dose and time [F(9,111)=10.8, p<0.0001] (Figure 5). The maximum effect of oxycodone occurred 15 min after administration and only the highest dose of 3.0 mg/kg produced an effect significantly greater than that of saline.

**Effects of intrathecal administration of analgesics on VTA ICSS in SNL rats.**

Analgesics known to reverse mechanical allodynia in SNL rats following intrathecal administration did not alter the EF50’s for VTA ICSS in SNL rats (Figure 6A). Administration of intrathecal saline did not change the EF50 for VTA ICSS in SNL rats [F(1,13)=0.4, p=0.6], nor did it alter the Rmax [F(1,13)=0.2, p=0.7]. Administration of intrathecal clonidine did not alter EF50 values at a dose of either 3 [F(1,13)=0.01, p=0.9] or 10 [F(1,13)=2.1, p=0.2] µg in SNL rats. The higher dose of 10 µg of clonidine decreased Rmax compared to pretreatment values [F(1,13)=7.4, p=0.02] while 3 µg had no effect [F(1,13)=2.6, p=0.1]. Gabapentin administration was without effect on either the EF50 [F(1,13)=0.08, p=0.8] or Rmax [F(1,13)=1.0, p=0.3]. Adenosine administration also did not affect
Figure 6. Effects of spinal analgesics on facilitation of electrical stimulation of the ventral tegmental area and on paw withdrawal thresholds (PWT) in spinal nerve-ligated (SNL) rats. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the three components following drug injection (4-6) from the EF50 for the two components preceding drug injection (2-3) for intrathecal clonidine (3 and 10ug), adenosine (30ug), and gabapentin (100ug). Data shown are averages across SNL (n=7) rats. (B) The effects of intrathecal clonidine (3 and 10ug), adenosine (30ug), and gabapentin (100ug) on PWT’s 60-min following intrathecal administration. Data shown are averages across SNL (n=7) rats. * Significantly different from saline treatment P ≤ 0.05.
the frequency-rate curve for VTA ICSS, producing no significant change in either
the EF50 [F(1,13)=0.1, p=0.4] or the Rmax [F(1,13)=0.5, p=0.5].

**Effect of intrathecal analgesics on PWT in SNL rats.**

All drugs given intrathecally reversed mechanical allodynia in SNL rats with
similar efficacy (Figure 6B). Saline had no effect on PWT after intrathecal
administration [F(1,13)=0.03, p=0.9] while both 3 and 10 µg of clonidine
significantly increased PWT compared to baseline values [3 µg: F(1,13)=11.1,
p=0.006; 10 µg: F(1,13)=65.3, p<0.0001]. Comparable effects were found
following intrathecal administration of 30 µg of adenosine [F(1,13)=17.0, p=0.001]
or 100 µg of gabapentin [F(1,13)=19.1, p=0.001].

**Effect of electrical stimulation of the VTA on PWT in SNL rats.**

Electrical stimulation applied to the left VTA did not significantly alter PWT in
SNL rats across a range of stimulation frequencies comparable to those used to
maintain ICSS (45 – 156 Hz) [F(5,41)=0.1, p=0.98] (Table 1). The stimulus
intensity applied to the VTA in this experiment was similar to that used in the
ICSS paradigm (158 ± 21 µA).
Table 1

Effects of Electrical Stimulation of the Ventral Tegmental Area on Mechanical Allodynia

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>PWT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.61 ± 0.30</td>
</tr>
<tr>
<td>45</td>
<td>2.90 ± 0.33</td>
</tr>
<tr>
<td>68</td>
<td>2.74 ± 0.33</td>
</tr>
<tr>
<td>103</td>
<td>2.92 ± 0.69</td>
</tr>
<tr>
<td>136</td>
<td>3.06 ± 0.55</td>
</tr>
<tr>
<td>156</td>
<td>2.90 ± 0.37</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (n=7)
PWT = paw withdrawal threshold
Discussion

Amidst the growing prescription opioid abuse problem, there is a clear need to understand how chronic pain alters the rewarding effects of commonly abused prescription opioids. The present data indicate that SNL reduces the potency of methadone, fentanyl, and hydromorphone in producing rewarding effects as measured using facilitation of VTA ICSS. Administration of spinal analgesics at doses that alleviated mechanical allodynia following SNL failed to facilitate VTA ICSS in SNL rats, indicating that alleviation of mechanical allodynia per se does not significantly stimulate dopaminergic pathways within the reward system. Electrical stimulation of the VTA at parameters similar to those experienced during VTA ICSS failed to reverse mechanical allodynia in SNL rats as well, further indicating that stimulation of reward pathways in the brain and reversal of mechanical allodynia are not interrelated. Drug effects on VTA ICSS in rats with peripheral nerve injury therefore appear to be related exclusively to abuse liability within the context of pain, unlike systemic drug self-administration or CPP in which both analgesic and positive reinforcing effects may have significant roles in the behavioral measures.

The ability of a drug to facilitate VTA ICSS has long been suggested to be indicative of its abuse potential. Therefore, a reasonable interpretation of the current data is that the abuse potential of prescription opioids is diminished following peripheral nerve injury. Previously we reported that morphine was ineffective in facilitating VTA ICSS following nerve injury, whereas heroin was still effective but less potent.\textsuperscript{18} Heroin is an illicit drug with a relatively high abuse
potential compared to morphine, attributed in part to the ability of heroin to more rapidly cross the blood brain barrier than morphine,\textsuperscript{21} where it is metabolized to 6-monoacetylmorphine (6-MAM),\textsuperscript{22} a metabolite that is more efficacious in stimulating $\mu$ opioid receptors than morphine.\textsuperscript{23} This led us to question if prescription opioids, with presumably lower abuse potential than heroin, would like morphine be ineffective in facilitating VTA ICSS following SNL. Surprisingly, each commonly abused prescription opioid, with the exception of oxycodone, shared a similar profile (reduced potency) to that previously reported with heroin, while morphine was again shown to be ineffective in facilitating VTA ICSS following nerve injury.\textsuperscript{18} The inability of oxycodone to potentiate VTA ICSS in SNL rats at the doses administered was unexpected, and may be due to sedative effects. Oxycodone was the only opioid administered that decreased maximal rates of responding in SNL rats, an effect often attributed to sedative effects or general disruption of behavior.

The fact that commonly abused prescription opioids produced effects similar to heroin using the VTA ICSS procedure is interesting given previous studies evaluating opioid self-administration in SNL rats. These experiments showed that methadone and heroin retain similar rates of self-administration in SNL compared to control rats, albeit at higher dose requirements, whereas self-administration rates of morphine, fentanyl, and hydromorphone are reduced following SNL compared to control subjects.\textsuperscript{7} Although there is considerable agreement between the present data on the effects of opioids on VTA ICSS and previous data on opioid self-administration in SNL rats, there are notable differences
between these paradigms with fentanyl and hydromorphone. Fentanyl and hydromorphone facilitate VTA ICSS in the present study, yet were found to be poor reinforcers in self-administration experiments in SNL rats. These discrepancies highlight the differences between these two behavioral paradigms. The subjective effects of electrical stimulation of a discrete population of cells in the brain are undoubtedly different than those produced following systemic administration of an opioid. Drug self-administration requires an animal to evaluate many factors pertinent to the subjective effects of the drug, including drug onset, duration of action, and potential rewarding/aversive effects associated with drug intake. Drug facilitation of ICSS, on the other hand, is a much more passive approach; drug administration is experimenter-delivered and the ability of the drug to alter a previously learned behavior serves as the primary dependent measure. Studies using both paradigms indicate that opioid reward is diminished in SNL rats, providing support for the complementary use of these two paradigms in understanding the interaction between the presence of pain and opioid addiction.

Another major finding of the current work was the inability of spinal analgesics to facilitate VTA ICSS in SNL rats. Given that administration of the spinal analgesic clonidine has previously been shown to elicit rewarding effects selectively in rats with neuropathic pain, it was hypothesized that alleviation of mechanical allodynia in nerve-injured rats could stimulate brain dopamine pathways, and subsequently facilitate VTA ICSS. This was not the case, as administration of intrathecal clonidine, adenosine, and gabapentin, at doses that
alleviated mechanical allodynia, failed to alter VTA ICSS. This suggests that alleviation of mechanical allodynia per se is not rewarding in the sense that it activates mesolimbic dopamine reward pathways. It also provides further support against the notion that activation of brain dopamine reflects a final pathway necessary in drug reinforcement, particularly in the context of pain. Previous studies assessing the rewarding effects of intrathecal clonidine using CPP and intrathecal self-administration both assessed the effects of 10μg clonidine, however this dose significantly reduced maximal responding for VTA ICSS. Decreases in maximal response rates associated with VTA ICSS can be difficult to interpret, and for this reason we tested a lower dose (3μg) that did not alter maximal responding or facilitate VTA ICSS, but still reversed mechanical allodynia.

In the present study we found that activation of limbic dopamine pathways by electrical stimulation of the VTA at similar parameters to those used during VTA ICSS failed to reverse established mechanical allodynia following SNL. This finding is consistent with previous reports that psychostimulants, which function predominantly to increase dopamine transmission, are effective in alleviating tonic (e.g., pain behaviors following formalin injection) but not phasic pain (e.g., tail-flick). SNL rats do not exhibit clear overt behavioral manifestations of ongoing pain, and due to this we could not test whether VTA stimulation alters ongoing pain following nerve injury.

The current data reveal that commonly abused prescription opioids stimulate classic reward pathways at similar doses to those that reverse mechanical
alldynia in rats following peripheral nerve injury. It is possible that the overlap in
effective doses indicates a direct relationship between opioid alleviation of
alldynia and stimulation of mesolimbic dopamine, such that one affects the
other. This is unlikely for several reasons. We previously reported that doses of
morphine that reversed mechanical alldynia did not facilitate VTA ICSS; doses
of cocaine that facilitated VTA ICSS did not alleviate mechanical alldynia in SNL
rats.\textsuperscript{18} Also, since spinal analgesics alleviated mechanical alldynia but failed to
facilitate VTA ICSS, and electrical stimulation of the VTA did not alter mechanical
alldynia, it is clearly possible to alleviate hypersensitivity without stimulating the
mesolimbic dopaminergic system, and vice-versa in SNL rats. Therefore, the
overlap in effective doses for reversing hypersensitivity and facilitating VTA ICSS
by opioids is unlikely due to one affecting the other. Future studies would be
helpful in this respect, by determining if the suppressive effects of nerve injury on
opioid facilitation of VTA ICSS can be prevented or reversed with nonopioid
treatments that prevent or reverse hypersensitivity or ongoing pain following
nerve injury.

The current data support the notion that prescription opioids are less effective
in activating brain reward pathways following peripheral nerve injury in rats, and
also suggest that effects on VTA ICSS are independent of reversal of mechanical
hypersensitivity. With abuse a major concern in prescribing opioids for pain, it is
ideal that the rewarding effects of opioids be suppressed in chronic pain states. It
should be concerning, however, that the suppressive effects of SNL on opioid
facilitation of VTA ICSS generally only reduced the potency of each opioid
assessed in the current study. Neuropathic pain patients often have higher opioid dose requirements than those used to treat acute nociceptive pain. In a double-blind study opioids were shown efficacious in treating neuropathic pain, however high doses produced significantly better reductions in reported pain intensity. High opioid dose requirements in neuropathic patients may reflect a diminished ability of opioids to either inhibit ascending noxious input or to activate brainstem descending pain modulation circuitry following neuropathy. It is also possible that stimulation of limbic dopamine modulates neuropathic patients' subjective pain scores, and that higher opioid dose requirements reflects decreased opioid stimulation of mesolimbic dopamine, similar to the decreased facilitation of VTA ICSS observed in SNL rats.

In conclusion, operant paradigms such as drug self-administration and VTA ICSS, along with conditioning experiments such as CPP, function as complementary preclinical tools for examining drug reinforcement mechanisms in the presence of pain. The use of all three strategies could assist in developing effective analgesics with diminished abuse potential, or in developing drugs that reduce the abuse liability of opioids without altering their analgesic properties.
Acknowledgements.

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland.
References


18. Ewan EE, Martin TJ: Opioid facilitation of rewarding electrical brain stimulation is suppressed in rats with neuropathic pain. Anesthesiology 2011; 114: 624-32


CHAPTER IV

SUPPRESSION OF REWARDING BRAIN STIMULATION AFTER PAW INCISION IN RATS

Eric E. Ewan and Thomas J. Martin

Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design.
Abstract

Stimulation of dopamine neurotransmission in the mesolimbic dopaminergic system is implicated in the rewarding effects of environmental stimuli and drugs of abuse. Conversely, aversive stimuli such as pain are thought to suppress activity of this system. Rats with or without spinal nerve ligation (SNL) were implanted with electrodes into the left ventral tegmental area (VTA) and trained to lever press for electrical stimulation (ICSS). Once responding was stable, the effects of paw incision surgery on VTA ICSS and on mechanical hypersensitivity around the incision site was assessed in control and SNL rats. Responding for VTA ICSS was similar in control and SNL rats. Paw incision reduced the frequency at which rats emitted 50% of maximal responding to a similar degree in control (maximal shift of -15.8 ± 2.1 Hz) and SNL (-11.8 ± 3.2 Hz) rats. Paw incision also reduced paw withdrawal thresholds around the incision similarly in control and SNL rats. The suppressive effects of paw incision on VTA ICSS was attenuated after 1d for both control and SNL rats, and when data was averaged across groups, was attenuated by 3d after surgery. On the other hand, mechanical hypersensitivity compared to baseline persisted for 10d following paw incision surgery, in both groups. The short duration of effect of paw incision on suppression of VTA ICSS suggests that spontaneous pain, but not hypersensitivity, suppresses mesolimbic dopamine neurons. To this end, suppression of VTA ICSS may represent a novel approach for measuring spontaneous pain in the postoperative period in animals.
Introduction

The mesolimbic dopaminergic system plays a well established role in reward. Dopamine neurons from the ventral tegmental area (VTA) project to the nucleus accumbens (NAcc), representing a key neural substrate underlying the reinforcing aspects of rewarding stimuli. (Pierce & Kumaresan, 2006) Growing evidence indicates that aversive stimuli such as pain depress VTA dopamine neurons, (Ungless, Magill, & Bolam, 2004).

Intracranial self-stimulation (ICSS) is a technique used to study the effects of environmental stimuli on limbic dopamine neurotransmission (Carlezon & Chartoff, 2007). In this paradigm rats lever press to receive brief electrical stimulation through an electrode implanted in a discrete brain region. Drugs such as opioid and dopamine agonists, which stimulate dopamine transmission, potentiate the reinforcing effects of VTA ICSS. (Wise, 1996) Conversely, aversive stimuli such as administration of intraperitoneal lactic acid, an acute noxious stimulus, suppresses the reinforcing effects of ICSS of the medial forebrain bundle, (Pereira Do Carmo et al., 2009) an axon bundle consisting of dopamine fibers from the VTA. This suggests that acute pain suppresses mesolimbic dopamine, which can be assessed using ICSS.

The paw incision model has been used extensively to study aspects of postoperative pain (Brennan, 2011). Following plantar incision rats exhibit thermal and mechanical hypersensitivity in the tissue surrounding the incision site which persists for five to ten days (Zahn & Brennan, 1999). Rats also exhibit signs of spontaneous pain, including guarding behavior which develops early in
the postoperative period and persists for two to three days (Brennan, Vandermeulen, & Gebhart, 1996), a time course which corresponds with increased spontaneous activity of Aδ and C fibers. (Pogatzki, Gebhart, & Brennan, 2002) This suggests that spontaneous pain following paw incision may be due to spontaneous activity of nociceptive pathways, an effect that is much shorter in duration compared to hypersensitivity.

The goal of the current study was to determine if postoperative pain inhibits the mesolimbic dopaminergic system by measuring the suppressive effects of paw incision on the reinforcing effects of VTA ICSS in rats. Following acquisition and stable responding for VTA ICSS, rats were subjected to paw incision surgery, and the effects on responding for VTA ICSS were evaluated. A second goal was to determine if prior and ongoing neuropathic pain modifies the effects of paw incision on VTA ICSS. Therefore, similar procedures were employed in rats previously subjected to a spinal nerve ligation (SNL). In addition, the effects of paw incision on paw withdrawal thresholds (PWT) of the area surrounding the incision was assessed in rats with and without neuropathic pain.
Methods

Subjects

Subjects were 17 male, Fisher 344 rats (9 Control and 8 SNL) weighing between 275-325 g at the beginning of the experiment (Harlan Laboratories, Raleigh, NC). Rats were group-housed in a temperature and humidity controlled room that was maintained on a reversed light-dark cycle (dark 05:00-17:00); this room was adjacent to the room in which behavioral experiments were performed. Food and water were continuously available except during behavioral experiments. All procedures were conducted according to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Surgeries

_Electrode Implantation_. Rats were permanently implanted with electrodes as previously described (Ewan & Martin, 2011). Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal) and received penicillin G procaine (75,000 U, intramuscular) to prevent infection. Rats were secured in a stereotaxic frame and platinum bipolar stimulating electrodes (Plastics One, Roanoke, VA) were aimed at the left VTA (2.1mm anterior to lambda, 0.6mm lateral from the midline, and 8.5mm), which were secured by three stainless steel screws embedded in dental acrylic on the skull surface.
Spinal Nerve Ligation. After electrode implantation 9 of the 17 rats were subjected to SNL as previously described. (Martin et al., 2007) Briefly, an incision was made through the skin and muscle of the lower back, and the left transverse process of the fifth lumbar vertebra was removed using bone microrongeurs. The fifth lumbar nerve was exteriorized and ligated with 4.0 silk suture. The sixth lumbar nerve was exteriorized from below the iliac bone at the sciatic notch and similarly ligated. Afterwards, muscle layers were sutured with 4.0 chromic gut, the skin with 4.0 nylon suture, and exterior wounds dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

Paw Incision. Rats were subjected to paw incision surgery as previously described (Brennan, Vandermeulen, & Gebhart, 1996). Briefly, rats were anesthetized with inhaled isoflurane (2%) in oxygen. A 1cm incision was made in the left hindpaw near the heel, the plantaris muscle lifted and incised longitudinally, and afterward the skin sutured with 4.0 nylon suture.

Paw Withdrawal Threshold

Mechanical allodynia was assessed using von Frey filaments (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL; Nichols et al., 1995) for all animals using Dixon non-parametric statistics.(Chaplan et al., 1994) PWT's of the mid-paw were assessed 14 days after SNL to verify development of allodynia. PWT's of the heel were also assessed prior to and in the days following paw incision surgery.
Intracranial Self-Stimulation (ICSS)

Apparatus. Operant chambers housed within sound- and light-attenuating enclosures equipped with a houselight and ventilation fan were used (Med Associates Inc., St. Albans, VT). These chambers have a lever 5 cm above a grid bar floor, stimulus lamp 2 cm above the lever, and a tone generator. An ICSS stimulator controlled by computer software (Med Associates Inc.) that controlled all stimulation parameters and data collection was located outside of the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One) positioned above the operant chamber connected the electrodes to the ICSS stimulator via 25cm cables (Plastics One).

Behavioral Procedure. Following at least 14 days recovery from electrode implantation, rats were trained to lever press for electrical stimulation of the VTA as previously described (Ewan & Martin, 2011). Illumination of the stimulus light indicated stimulus availability and each lever press generated a 0.5-sec train of rectangular alternating cathodal and anodal pulses (0.1-ms pulse durations). During stimulation the stimulus light shut off, the houselight turned on, and a tone sounded. Responding during stimulation resulted in no further stimulation and was not recorded.

During training sessions the frequency was held constant (150Hz) and the intensity adjusted to maintain consistent responding. Following this initial training frequency-response curves were generated. These 90-min sessions consisted of six 10-min components. Each component consisted of ten 60-sec trials. Each trial began with a 5-sec timeout followed by a 5-sec priming period in which rats
received five noncontingent stimulations, and concluded with a 50-sec response period. Current intensity remained constant (unique to each animal) and 10 frequencies (156-45 Hz, 0.06 log increments) corresponding to each trial were made available in descending order. A 30-min timeout separated the third and fourth components. For data analysis, components 2 and 3 were averaged and analyzed with Prism software (sigmoidal-dose response, variable slope; Graph Pad, La Jolla, CA). ICSS sessions were conducted daily and once responding was stable, rats were anesthetized with isoflurane 4-hr prior to an ICSS session. The following day rats were anesthetized with isoflurane and were subjected to paw incision surgery. Daily ICSS sessions continued in the days after surgery. Experimental sessions in which the baseline and post-incision results were obtained were performed between 1-3 months after initial surgery.

**Histology**

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed, frozen in isopentane (-35°C), and stored at -80°C. Coronal sections (25μm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the VTA (Figure 1).

**Data Analysis**

Data for PWT’s was analyzed using a two-way ANOVA with group and time after incision serving as the independent variables. The EF50 (frequency at which rats emitted 50% of maximal responding) and maximum response rate for VTA ICSS was calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad). The effect of paw incision on VTA ICSS was
analyzed using a two-way ANOVA with group and time after incision serving as the independent variables and $\Delta$EF50 or maximal response rates serving as the dependent measures. Post-hoc analyses within the control or SNL groups were made using Dunnett's t-test for multiple comparisons with isoflurane treatment serving as control. A two-tailed p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc., Cary, NC).
Figure 1. Schematic showing the location of stimulating electrodes within the ventral tegmental area for control and spinal nerve-ligated (SNL) rats. Numbers next to diagrams indicate the distance anterior to the interaural line according to the atlas of Paxinos and Watson (1998).
Control

3.8 mm

SNL

3.2 mm
Results

Effects of paw incision on mechanical thresholds in control and SNL rats.

Paw incision surgery significantly decreased mechanical thresholds in a time-dependent manner in both control [F(9,89)=68.3, p<0.0001] and SNL [F(9,79)=46.6, p<0.0001] rats. (Figure 2) The baseline PWT at the incision site prior to incision was significantly less in SNL rats compared to control [F(1,16)=6.8, p=0.02], but not 4 hr after paw incision [F(1,16)=0.9, p=0.4]. In control rats, PWT began to recover on day 7 and was not different from pre-surgery baseline values on day 14 after incision. In SNL rats, the recovery time course was similar with the PWT being significantly greater on day 7 than at 4 hr after surgery and returning to pre-surgery baseline values on day 14.

Effects of paw incision on VTA ICSS in control and SNL rats.

Baselines values did not differ between control and SNL animals for either the EF50 [F(1,16)=0.2, p=0.7] or maximal response rates [F(1,16)=0.9, p=0.3]. Exposure to isoflurane anesthesia alone had no effect on either the EF50 or maximal response rate for VTA ICSS in control [F(1,17)=0.03, p=0.9; F(1,17)=0.5, p=0.5, respectively] or SNL [F(1,15)=0.01, p=0.9; F(1,15)=0.01, p=0.9, respectively] rats (Figure 3A). In control rats, paw incision surgery significantly shifted the frequency-rate curve for VTA ICSS to the right, significantly increasing the EF50 [F(4,44)=3.4, p=0.02] while having no effect on maximal response rates [F(4,44)=1.0, p=0.4] (Figure 3B). The effect of paw incision lasted for only 4 hr however, as the EF50 values were no different from
Figure 2. Mechanical hypersensitivity around the incision site following paw incision in control and spinal nerve-ligated (SNL) rats. Paw withdrawal thresholds (PWT) of the heel were assessed before and after (4-hr and 14 days) paw incision surgery using von Frey filaments in control and SNL rats. * Significantly different from baseline P ≤ 0.05 # Significantly different from 4-hr. P ≤ 0.05 α Significantly different from control. P ≤ 0.05
Figure 3. Effects of paw incision on responding for electrical stimulation of the ventral tegmental area (VTA ICSS) in control and spinal nerve-ligated (SNL) rats. Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for each test session from the EF50 for the baseline session. Data shown are averages across control (n=9) and SNL (n=8) rats. Frequency-response curves for baseline and 4-hr after paw incision are shown for control (B) and SNL (C) rats. * Significantly different from baseline P ≤ 0.05.
A Effects of Paw Incision on VTA ICSS

B Control

C SNL
baseline 24 hr after paw incision (p≥0.05). The effect of paw incision in SNL rats on VTA ICSS was similar, with paw incision increasing the EF50 compared to baseline values [F(4,39)=3.2, p=0.03] while having no effect on maximal responding [F(4,39)=1.3, p=0.3]. (Figure 3C) As in the control group, paw incision only increased the EF50 for VTA ICSS 4 hr after surgery in SNL rats, with the EF50 returning to baseline values 24 hr after surgery (p≥0.05). (Figure 3) The effect of paw incision surgery was not different between control and SNL rats at the 4 hr time point, with surgery increasing the EF50 to a similar extent in each group [F(1,16)=0.3, p=0.6].

Since no differences were observed between control and SNL rats for the effects of paw incision on EF50 or maximal responding at any time point, data from VTA ICSS sessions before and after incision were averaged across groups and analyzed. When analyzed as a single group, paw incision surgery significantly increased EF50 for VTA ICSS for up to 2 days after surgery across groups F(4,84)=6.5, p<0.0001] while having no effect on maximal responding [F(4,84)=2.0, p=0.1]. (Figure 4).
Figure 4. Effects of paw incision on responding for electrical stimulation of the ventral tegmental area (VTA ICSS) in control and spinal nerve-ligated (SNL) rats. Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for each test session from the EF50 for the baseline session. Data across control and SNL rats (n=17) was averaged. * Significantly different from saline treatment $P \leq 0.05$. 
Discussion

The current study sought to determine if postoperative pain inhibits the mesolimbic dopaminergic system by measuring the suppressive effects of paw incision on the reinforcing effects of VTA ICSS in control and SNL rats. In both groups paw incision reduced responding for VTA ICSS to a similar extent. The effect of paw incision surgery on VTA ICSS for each group was significant only at the 4-hr time point after surgery; when data was averaged across groups these suppressive effects endured for 2 days post incision. Paw incision also produced mechanical hypersensitivity around the incision site in both groups to a similar degree, but the hypersensitivity didn’t begin to recover until day 7, and was still significantly different from baseline 10 days after incision. The different time courses of effect for which paw incision inhibited VTA ICSS and produced mechanical allodynia suggest that the two are unrelated, and that mechanisms other than hypersensitivity mediate suppression of mesolimbic dopamine in the postoperative period.

The effect of paw incision on suppression of VTA ICSS is likely related to ongoing spontaneous pain immediately after incision. The relatively short time course of effect on VTA ICSS is similar in duration to guarding behavior exhibited following paw incision (Brennan et al., 1996), which also corresponds to the timeframe in which nociceptors exhibit increased spontaneous activity following incision (Pogatzki et al., 2002; Xu & Brennan, 2009). Therefore, suppression of VTA ICSS by paw incision likely results from increased spontaneous activity of nociceptive pathways that inhibit mesolimbic dopamine neurotransmission.
One possible source for pain suppression of mesolimbic dopamine is the ascending spinoparabrachial pain pathway. The parabrachial nucleus (PBN) projects to the VTA, and intra-PBN lidocaine attenuates noxious foot shock-induced inhibition of VTA dopamine neurons. Similarly, the amygdala may play a role in pain suppression of mesolimbic dopamine. The amygdala receives direct ascending nociceptive input from the PBN (Bernard & Besson, 1990) and can modulate activity of VTA dopamine neurons through GABAergic projections to the VTA (Everitt et al., 1999) as well as glutamatergic projections to the nucleus accumbens (Brog et al., 1993; Wright et al., 1996).

Neuropathic pain did not further exacerbate the suppressive effects of paw incision on VTA ICSS. This was surprising given that neuropathy produces significant peripheral and central sensitization (Suzuki & Dickenson, 2005). Sensitization of pain pathways following nerve injury predicts that SNL rats would demonstrate a hyperalgesic effect to paw incision, yet the suppressive effects of paw incision were similar in magnitude and duration in SNL rats. Similarly, mechanical hypersensitivity around the incision site was not different in SNL or control rats, though interpretation of this is complicated by the fact that SNL rats had lower baseline PWT’s than uninjured rats, and the degree of hypersensitivity immediately following paw incision was large enough that any differences between groups would likely be indistinguishable due to a floor effect. In contrast, baseline EF50’s between SNL and control rats were similar for VTA ICSS, which is similar to previous findings showing that SNL does not alter baseline responding for VTA ICSS (Ewan & Martin, 2011). One implication of this is that
SNL does not produce spontaneous pain of sufficient intensity to affect VTA ICSS. Similarly, nerve injury does not appear to alter the magnitude or duration of spontaneous pain following paw incision as measured with VTA ICSS.

Future research aimed at reversing the suppressive effects of paw incision on VTA ICSS would be helpful in understanding how ongoing pain suppresses the mesolimbic dopamine system. This could include peripheral nerve block or spinal analgesics to block ascending nociceptive input. Furthermore, intracranial drug injection studies would be helpful in determining the underlying nociceptive circuits responsible for suppression of mesolimbic dopamine following incision. Anatomically, input from the PBN and amygdala are prominent candidates to study in this regard.

In conclusion, paw incision surgery produced mechanical allodynia around the incision site and suppressed the reinforcing effects of VTA ICSS to a similar degree in control and SNL rats. The time course of effects differed, with suppression of VTA ICSS being much shorter in duration compared to mechanical hypersensitivity following paw incision. The short duration of effect of paw incision on suppression of VTA ICSS suggests that spontaneous pain, but not hypersensitivity, suppresses mesolimbic dopamine neurons, which may be mediated through increased spontaneous activity of ascending spinoparabrachial neurons. Therefore, suppression of VTA ICSS may represent a novel technique for measuring spontaneous pain in the postoperative period.
Acknowledgements.

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland.
References


CHAPTER V

INTRACRANIAL SELF-STIMULATION OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS: EVIDENCE FOR OXYTOCIN-MEDIATED REINFORCEMENT MECHANISMS

Eric E. Ewan and Thomas J. Martin

The following chapter is in preparation for submission to Anesthesiology, and is reprinted with permission. Stylistic variations are due to conforming to the publishing journal. Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design and manuscript preparation.
Abstract

Introduction. Continued interest exists in developing behavioral measures assessing spontaneous pain following nerve injury. Intracranial self-stimulation (ICSS) may be useful in this regard, by measuring the reinforcing effects of electrical stimulation of brain regions implicated in pain relief.

Methods. Control and spinal nerve ligated (SNL) rats were implanted with electrodes into the PVN or medial forebrain bundle (MFB). The effect of electrical stimulation of the PVN or MFB on mechanical allodynia was assessed in SNL rats. Control and SNL rats were trained to lever press for PVN or MFB ICSS, and pharmacological studies explored the involvement of oxytocin or opioid mechanisms.

Results. Electrical stimulation of the PVN reversed mechanical allodynia in SNL rats and was reinforcing in both control and SNL rats at frequencies similar to those that alleviated mechanical allodynia. Intrathecal atosiban significantly increased the frequency at which rats emitted 50% of maximal responding for PVN ICSS in SNL (maximal shift 13.76 ± 3.35 Hz) but not control (9.61 ± 4.05 Hz) rats. Intrathecal naltrexone or clonidine, as well as intraperitoneal atosiban or naltrexone did not alter PVN ICSS in SNL rats. Electrical stimulation of the MFB had no effect on mechanical allodynia in SNL rats, and intrathecal atosiban did not alter MFB ICSS in either control or SNL rats.

Conclusions. Electrical stimulation of the PVN produces both antiallodynic and reinforcing effects in SNL rats that may be partially mediated by spinal release of
oxytocin. Surprisingly, electrical stimulation of the PVN produces reinforcement in normal rats as well.
Introduction

Assessment of neuropathic pain in animals often utilizes reflexive measures such as withdrawal from a noxious stimulus as the primary dependent measure.\(^1\) Neuropathic patients’ primary complaint is spontaneous pain,\(^2\) which has led to increased interest in developing behavioral measures that assess pain in the absence of an external noxious stimulus. Motor cortex stimulation produces conditioned place preference selectively in rats with spinal cord lesions,\(^3\) suggesting that stimulation of brain regions that alleviate ongoing pain may be reinforcing in neuropathic rats. Intracranial self-stimulation (ICSS), a behavioral paradigm that pairs operant behavior (e.g., lever presses) with brief electrical stimulation of discrete brain regions, has been used extensively in studying the reinforcing effects of brain reward systems,\(^4\) and therefore represents one potential avenue for studying the reinforcing effects of brain stimulation that alleviates ongoing pain. Importantly, information about the reinforcing effects of brain stimulation mediated by alleviation of ongoing pain in neuropathic rats may provide novel targets for treating neuropathic pain in humans.

The paraventricular nucleus (PVN) of the hypothalamus contains oxytocinergic neurons that are capable of altering pain transmission. PVN neurons containing oxytocin project throughout the CNS,\(^5\) including a direct projection to the spinal cord.\(^6\) Electrical stimulation of the PVN releases oxytocin in CSF, plasma, and spinal cord,\(^7\) and stimulation alleviates mechanical allodynia following nerve injury that is reversed by spinal administration of an oxytocin receptor antagonist and partially reversed by spinal administration of an opioid
Exogenous oxytocin produces analgesia following systemic, spinal, and intracerebroventricular delivery in rats, indicating possible pain modulation at central or peripheral sites. Pharmacological stimulation of the PVN causes release of endogenous opioid peptides in the spinal cord, supporting a potential role for both oxytocin and endogenous opioids in mediating analgesia induced by electrical stimulation of the PVN.

The current study explored the possibility that stimulation of the PVN, which is implicated in pain relief, may produce reinforcing effects in rats with neuropathic pain through release of oxytocin or endogenous opioids. One goal of this study was to assess the ability of electrical stimulation of the PVN to reverse mechanical allodynia in nerve-injured rats. A second goal was to determine if PVN stimulation produces reinforcing effects that are selective for or enhanced in rats with nerve injury. Therefore, the reinforcing effects of PVN stimulation were assessed using the ICSS paradigm (PVN ICSS) in rats with and without neuropathic pain. A third goal was to determine if the anti-allodynic and reinforcing effects of PVN stimulation are mediated by oxytocinergic and opioidergic mechanisms. Therefore, pharmacology studies targeting oxytocin and opioid receptors were undertaken, and the effects on the anti-allodynic as well as reinforcing effects of PVN stimulation were assessed. Lastly, given the proximity of the PVN to the medial forebrain bundle (MFB), an axon bundle in the lateral hypothalamus that supports ICSS (MFB ICSS), similar manipulations were performed with electrical stimulation of the MFB as a control.
Materials and Methods

Subjects

Subjects were 39 male, Fisher 344 rats (12 SNL and 11 control rats for PVN experiments; 9 SNL and 7 control rats for MFB experiments) weighing between 275-325 g at the beginning of the experiment (Harlan Laboratories, Raleigh, NC). Rats were group-housed in a temperature and humidity controlled room that was maintained on a reversed light-dark cycle (dark 05:00-17:00); this room was adjacent to the room in which behavioral experiments were performed. Food and water were continuously available except during behavioral experiments. All procedures were conducted according to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Surgeries

Electrode Implantation. Rats were permanently implanted with electrodes as previously described. Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal) and received penicillin G procaine (75,000 U, intramuscular) to prevent infection. Rats were secured in a stereotaxic frame and platinum bipolar stimulating electrodes (Plastics One, Roanoke, VA) were aimed at the left PVN or left MFB (2.5mm; 3.0mm posterior to bregma, 0.3mm; 1.7mm lateral from the midline, and 8.0mm; 8.5mm below the skull, for PVN; MFB, respectively), which were secured by three stainless steel screws embedded in dental acrylic on the skull surface.
Spinal Nerve Ligation. After electrode implantation 21 of the 39 rats were subjected to SNL as previously described. Briefly, an incision was made through the skin and muscle of the lower back, and the left transverse process of the fifth lumbar vertebra was removed using bone microrongeurs. The fifth lumbar nerve was exteriorized and ligated with 4.0 silk suture. The sixth lumbar nerve was exteriorized from below the iliac bone at the sciatic notch and similarly ligated. Afterwards, muscle layers were sutured with 4.0 chromic gut, the skin with 4.0 nylon suture, and exterior wounds dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

Intrathecal Catheter Implantation. Rats were anesthetized in a similar manner to that used during electrode implantation and received intrathecal catheters as previously described. Rats were secured in a stereotaxic frame, the head bent downward, and an incision was made through the skin and muscle of the neck. A small hole was made in the atlanto-occipital membrane, and an 8.5 cm catheter was inserted and secured to the surrounding muscle with 5.0 Vicryl suture (Ethicon Inc., Cornelia, GA). Catheters were constructed from 32 g polyethylene tubing (ReCathCo, Allison Park, PA) fused to biocompatible Tygon tubing (inner diameter 0.01”, formulation S-54-HL, Saint-Gobain Plastics Inc., Akron, OH) using cyclohexanone. The skin was then sutured with 4.0 nylon suture and exterior wounds dressed with antibiotic powder.

Paw Withdrawal Threshold
Mechanical allodynia was assessed using von Frey filaments (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL)\textsuperscript{15} for all animals using Dixon non-
parametric statistics.\textsuperscript{16} PWT’s were assessed 14 days after surgery to verify development of allodynia. Experimenter-delivered electrical stimulation (30 0.5sec stimulations per minute) of the PVN or MFB was delivered using similar parameters to those during self-stimulation sessions. Specific testing procedures were used to prevent motor abnormalities during testing. Baseline PWT’s were determined prior to stimulation. With the frequency set to 50 Hz, the current was gradually increased (10uA increments) up to that used during self-stimulation sessions (unique to each rat; some rats exhibited motor effects and lower intensities than those used during self-stimulation were used). PWT’s were assessed to various frequencies in ascending order (50, 79, 112, 158 Hz for the PVN; 50, 78, 118, 156 Hz for the MFB). The frequency of stimulation was gradually increased (10Hz increments until the next scheduled test frequency) after each PWT assessment. Testing lasted between 8-12 minutes per rat.

\textbf{Drugs}

Morphine sulfate was purchased as a 15 mg/ml sterile solution (Baxter Healthcare; Deerfield, IL). Atosiban acetate was purchased from Selleck Chemicals LLC (Houston, TX). Naltrexone hydrochloride and clonidine hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO). All drugs were diluted using 0.9% (wt/vol) saline, pH 7.4.

\textbf{Intracranial Self-Stimulation (ICSS)}

\textit{Apparatus}. Operant chambers housed within sound- and light-attenuating enclosures equipped with a houselight and ventilation fan were used (Med Associates Inc., St. Albans, VT). These chambers have a lever 5 cm above a
grid bar floor, stimulus lamp 2 cm above the lever, and a tone generator. An ICSS stimulator controlled by computer software (Med Associates Inc.) that controlled all stimulation parameters and data collection was located outside of the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One) positioned above the operant chamber connected the electrodes to the ICSS stimulator via 25cm cables (Plastics One).

**Behavioral Procedure.** Following at least 14 days recovery from surgery, rats were trained to lever press for electrical stimulation of the PVN or MFB as previously described\(^\text{13}\). Illumination of the stimulus light indicated stimulus availability and each lever press generated a 0.5-sec train of rectangular alternating cathodal and anodal pulses (0.1-ms pulse durations). During stimulation the stimulus light shut off, the houselight turned on, and a tone sounded. Responding during stimulation resulted in no further stimulation and was not recorded.

During training sessions the frequency was held constant (150Hz) and the intensity adjusted to maintain consistent responding. Following initial training, frequency response curves were generated. For PVN ICSS, 120-min sessions consisted of five 18-min components. Each component consisted of eight 135-sec trials. Each trial began with a 5-sec timeout followed by a 10-sec priming period during which rats received five noncontingent stimulations, and concluded with a 120-sec response period. Current intensity remained constant (unique to each animal) and 8 frequencies (158-71 Hz, 0.05 log increments; PVN) corresponding to each trial were made available in descending order. A 30-min
timeout between the third and fourth components permitted drug injections. MFB ICSS was similarly performed with the following differences: 90-min sessions consisted of six 10-min components, which consisted of ten 60-sec trials. Each trial began with a 5-sec timeout, followed by a 5-sec priming period, and concluded with a 50-sec response period; 10 frequencies (156-45 Hz, 0.06 log increments) corresponded to each trial. At the beginning of the timeout rats received 1 mL/kg intraperitoneal injections of saline (0.9% wt/vol), naltrexone (1mg/kg), or atosiban (10 mg/kg); similarly rats received 20uL intrathecal injections (5uL drug followed by 15uL saline flush) of saline (0.9% wt/vol), atosiban (6-12ug), naltrexone (3ug), morphine (2ug), or clonidine (5ug). For data analysis, the 2 components preceding drug injection and the 2 (PVN) or 3 (MFB) components following drug injection were averaged and compared using Prism software (sigmoidal-dose response, variable slope; Graph Pad, La Jolla, CA). Test sessions were separated by at least 1 day. Sessions using intraperitoneal injection of test compounds were performed first. Rats were then implanted with intrathecal catheters, allowed 14 days for recovery, and subsequently tested with intrathecal drug manipulations. Test sessions were performed 1-4 months after initial surgery.

Histology

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed, frozen in isopentane (-35°C), and stored at -80°C. Coronal sections (25μm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the PVN or MFB (Figure 1).
Figure 1. Location of the stimulating electrodes within the paraventricular nucleus of the hypothalamus and medial forebrain bundle for control and spinal nerve-ligated (SNL) rats. Numbers left of each brain section indicate distance posterior to bregma, according to the atlas of Paxinos and Watson.21
Control

8.0 mm

7.6 mm

7.2 mm

6.7 mm

SNL
Data Analysis

Data for PWT’s was analyzed using a two-way ANOVA with drug dose and frequency of stimulation serving as the independent variables. The EF50 (frequency at which rats emitted 50% of maximal responding) and maximum response rate for PVN or MFB ICSS were calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad, San Diego, CA). The effect of drug treatment on PVN or MFB ICSS was analyzed using a one-way ANOVA with drug dose serving as the independent variables and ΔEF50 (EF50 prior to injection – EF50 after injection) or maximal response rates (RMax) serving as the dependent measures. Post-hoc analyses within the control or SNL groups were made using Dunnett’s t-test for multiple comparisons with saline injection serving as control. A two-tailed p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc., Cary, NC).
Results

Effects of electrical stimulation of the PVN on PWT in SNL rats.

Electrical stimulation of the PVN produced a frequency dependent anti-allodynic effect \[ F(4,39)=24.9, \ p<0.0001 \], with frequencies of 112 and 158 Hz increasing PWT compared to baseline values \(2.7\pm0.1\)g (Figure 2). PWT returned to baseline values at 5 and 10 min following cessation of PVN stimulation \(p\leq0.05\). Administration of 12 µg of atosiban intrathecally significantly attenuated the effects of PVN stimulation on PWT compared to saline treatment \[ F(1,79)=29.4, \ p<0.0001 \] and there was a significant interaction between treatment and frequency of stimulation \[ F(4,79)=6.9, \ p<0.0001 \], with atosiban significantly reducing the effects of both 112 and 158 Hz on PWT (Figure 2A). Administration of 10 mg/kg of atosiban intraperitoneally had no effect on the anti-allodynic effects of PVN stimulation \[ F(1,79)=3.3, \ p=0.08 \] (Figure 2A). Administration of either 3 µg of naltrexone intrathecally \[ F(1,79)=2.7, \ p=0.11 \] or 1 mg/kg of naltrexone intraperitoneally \[ F(1,79)=0.4, \ p=0.5 \] had no effect on the anti-allodynic effects of PVN stimulation compared to saline treatment (Figure 2B).

Effects of electrical stimulation of the MFB on PWT in SNL rats.

Electrical stimulation of the MFB failed to produce a significant effect on PWT over a range of 50 – 156 Hz \[ F(4,39)=1.2, \ p=0.3 \], with PWT not being significantly different from baseline values \(2.8 \pm 0.3 \)g at any frequency (intensity = 189.4 ± 12.1 µA) (Table 1).
Figure 2. Effects of electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) on paw withdrawal thresholds (PWT) in spinal nerve-ligated rats (SNL). The effects of 30 minute pretreatment on frequency-effect curves for PVN stimulation were determined for intrathecal saline (A, B), 10mg/kg intraperitoneal and 12ug intrathecal atosiban (A) as well as 1mg/kg intraperitoneal and 3ug intrathecal naltrexone (B) on PWT in SNL rats (n=8). * Significantly different from baseline. # Significantly different from saline  P ≤ 0.05.
A. Effects of Atosiban on PVN Stimulation

- **Saline**
- **10mg/kg Atosiban**
- **12ug Atosiban**

B. Effects of Naltrexone on PVN Stimulation

- **Saline**
- **1mg/kg Naltrexone**
- **3ug Naltrexone**
Table 1

*Effects of Electrical Stimulation of the Medial Forebrain Bundle on Mechanical Allodynia*

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>PWT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.75 ± 0.25</td>
</tr>
<tr>
<td>50</td>
<td>2.96 ± 0.30</td>
</tr>
<tr>
<td>78</td>
<td>3.38 ± 0.28</td>
</tr>
<tr>
<td>118</td>
<td>3.70 ± 0.50</td>
</tr>
<tr>
<td>156</td>
<td>3.83 ± 0.67</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (n=8)
PWT = paw withdrawal threshold
Effects of SNL on ICSS of the PVN or MFB.

The intensities used for ICSS were not significantly different between SNL and control groups for either the PVN \([F(1,22)=0.2, p=0.6]\) or the MFB \([F(1,15)=0.05, p=0.8]\). The maximum response rates also did not differ between control and SNL rats for ICSS in the PVN \([F(1,22)=0.2, p=0.6]\) or in the MFB \([F(1,15)=3.7, p=0.07]\) (Figure 3). The EF50 values did not differ between SNL and control rats for ICSS in the MFB \([F(1,15)=0.2, p=0.7]\), however the EF50 value for ICSS in the PVN was slightly but significantly greater in SNL rats (114.7±1.8) compared to control subjects (109.1±1.8) \([F(1,22)=4.6, p=0.04]\).

Comparison of ICSS parameters in the PVN and MFB.

The intensities used for ICSS did not differ (SNL and control rats combined) between the PVN and MFB \([F(1,38)=2.1, p=0.16]\). The maximum response rate maintained by ICSS in the PVN (23.8±1.1 resp/min) was approximately half that maintained in the MFB (45.1±1.3 resp/min) \([F(1,38)=160, p<0.0001]\) (Figure 3). The EF50 was also significantly greater for ICSS in the PVN (112.0±1.6) compared to the MFB (96.0±2.0) \([F(1,38)=39, p<0.0001]\).

Effects of intrathecal atosiban, naltrexone, morphine, and clonidine on PVN and MFB ICSS.

In SNL rats, intrathecal administration of saline had no effect on either the EF50 or Rmax for PVN ICSS. Intrathecal administration of 12 µg of atosiban however significantly shifted the rate-frequency curve to the right while administration of 6 µg of atosiban, naltrexone (3 µg), morphine (2 µg) or clonidine (5 µg) was without effect \([F(5,51)=3.4, p=0.001]\) (Figure 4A). Administration of
Figure 3. Baseline responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. Frequency-response curves for baseline responding were generated by averaging the second and third components preceding saline administration (prior to any drug treatments). The y-axis indicates the number of self-stimulations (0.5-sec) per 60 seconds for each frequency (x-axis). Data shown are averages across control (n=11, n=7) and SNL (n=12, n=9) rats with PVN and MFB electrodes, respectively. Average current intensities during baseline responding were 246.82 (17.51) uA and 215.71 (26.87) uA for control and 234.58 (19.59) uA and 208.33 (18.86) uA for SNL rats with PVN and MFB electrodes, respectively.
Figure 4. Effects of intrathecal atosiban, naltrexone, morphine and clonidine on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. (A, B) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the two (PVN) and three (MFB) components following drug injection from the EF50 for the two components preceding drug injection. (A) Intrathecal saline, atosiban (6 and 12ug), morphine (2ug), naltrexone (3ug), and clonidine (5ug) was administered to SNL rats with PVN electrodes (n=9 per drug except n=7 for clonidine). (B) Intrathecal atosiban (12ug) was administered to control (n=8, n=?) and SNL (n=9, n=8) rats with PVN and MFB electrodes, respectively. Frequency-response curves before and after 12ug atosiban (30-min) are shown for SNL rats with PVN (C, n=9) and MFB (D, n=8) electrodes.

* Significantly different from saline treatment P ≤ 0.05.
A 30-min Incubation: PVN SNL

B 30-min Incubation

C 12ug Atosiban: PVN SNL

D 12ug Atosiban: MFB SNL
either saline or 12 µg of atosiban intrathecally had no significant effect on PVN ICSS in control subjects on either the EF50 \[F(1,13)=2.4, p=0.15\] or the Rmax \[F(1,13)=2.5, p=0.15\] (Figure 4B). The effect of intrathecal administration of 12 µg of atosiban on ICSS in SNL rats was restricted to the PVN, as this manipulation did not alter either the EF50 \[F(1,15)=1.3, p=0.3\] or Rmax \[F(1,15)=0.1, p=0.8\] values for ICSS of the MFB in SNL rats (Figure 4B). Similarly 12 µg of atosiban intrathecally had no significant effect on MFB ICSS in control subjects on either the EF50 \[F(1,13)=0.9, p=0.4\] or the Rmax \[F(1,13)=1.6, p=0.2\] (Figure 4B).

**Effects of systemic administration of atosiban and naltrexone on PVN and MFB ICSS.**

Administration of saline intraperitoneally had no effect on either the EF50 or the Rmax for ICSS of the PVN or MFB in control or SNL rats. Administration of 1 mg/kg of naltrexone or 10 mg/kg of atosiban had no effect on either the EF50 \[F(2,28)=1.7, p=0.2\] or Rmax \[F(2,28)=1.3, p=0.3\] for ICSS of the PVN compared to saline treatment in SNL rats, and was also without effect on either the EF50 \[F(2,24)=1.6, p=0.2\] or Rmax \[F(2,24)=0.001, p=1.0\] for ICSS of the PVN in control rats (Figure 5). Similarly, intraperitoneal injection of naltrexone or atosiban had no effect on either the EF50 \[F(2,24)=2.1, p=0.15\] or Rmax \[F(2,24)=0.6, p=0.5\] for ICSS of the MFB in SNL rats, and was also without effect on either the EF50 \[F(1,12)=0.3, p=0.6\] or Rmax \[F(1,12)=1.8, p=0.2\] in control subjects (Figure 5).
Figure 5. Effects of intraperitoneal atosiban and naltrexone on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the two (PVN) and three (MFB) components following drug injection from the EF50 for the two components preceding drug injection. Atosiban (10mg/kg) or naltrexone (1mg/kg) was administered intraperitoneally to control (n=7) and SNL (n= 8, except n=9 for SNL PVN naltrexone) rats with PVN and MFB electrodes.
Discussion

Interest in measuring spontaneous pain in animals has led to increased development and use of behavioral measures assessing pain in the absence of an external noxious stimulus; this was pursued in the current study using ICSS. Electrical stimulation of the PVN alleviated mechanical allodynia and produced reinforcing effects at similar frequencies in SNL rats. Surprisingly, control rats also lever pressed for PVN ICSS at similar rates and frequencies to SNL rats. Pharmacology studies revealed that spinal release of oxytocin might mediate the reinforcing effects of PVN ICSS, which may interact with both reward and analgesic systems. Pharmacologically, these effects appear to be distinct from the reinforcing effects of MFB ICSS.

Previous work implicates spinal release of oxytocin and endogenous opioids in mediating the anti-allodynic effects of PVN stimulation. In the current study spinal atosiban partially reversed the anti-allodynic effects of PVN stimulation in SNL rats. However, spinal naltrexone was without effect, suggesting that oxytocin release plays a greater role in the antiallodynic effects of PVN stimulation than opioids. Though this may seem at odds with previous work, it is worth noting that when spinal opioid antagonism has been shown to reverse the anti-allodynic effects of PVN stimulation, the effects were smaller in magnitude compared to oxytocin receptor antagonists, and in some cases reversal by opioid antagonists have not been demonstrated. Stimulation of the PVN also induces oxytocin release in the periphery, and systemic administration of oxytocin has been shown to produce analgesia in rats. Systemic release of
oxytocin, however, does not appear to underlie the anti-allodynic effects of PVN stimulation, since systemic atosiban did not diminish the ability of PVN stimulation to reverse mechanical hypersensitivity. Systemic naltrexone was similarly without effect, further suggesting a diminished role of opioids in mediating the anti-hypersensitivity effects of PVN stimulation.

The similarities between the frequencies with which PVN electrical stimulation alleviated mechanical allodynia and maintained ICSS in SNL rats suggests that relief of hypersensitivity and/or ongoing pain mediates the reinforcing effects of PVN ICSS in SNL rats. However, two important findings from the current work question this interpretation. First, control rats responded for PVN ICSS at rates and frequencies similar to SNL rats, indicating that PVN stimulation produces effects other than analgesia that are reinforcing under normal conditions. It is therefore possible that the mechanisms responsible for PVN ICSS in control rats are shared with SNL rats. Second, administration of the spinal analgesic clonidine failed to alter the reinforcing effects of PVN ICSS in SNL rats. If PVN ICSS in SNL rats was mediated primarily by reversal of mechanical allodynia, one would expect that relief of allodynia (and possibly ongoing pain) with clonidine would diminish responding for PVN ICSS in SNL rats.

It was surprising that electrical stimulation of the PVN produced reinforcing effects in control rats. One possible explanation is that electrical stimulation of the PVN unintentionally stimulated the MFB. The MFB is an axon bundle in the lateral hypothalamus connecting dopamine cell bodies in the VTA to the nucleus accumbens (NAcc). Stimulation of the MFB increases dopamine release in the
NAcc and is reinforcing in rats. To investigate the possibility that inadvertent stimulation of the MFB mediates the reinforcing effects of PVN ICSS, control and SNL rats were implanted with electrodes in the MFB. Rats with MFB electrodes acquired lever pressing at a faster rate (data not shown), exhibited higher response rates and generally had lower intensity and frequency requirements than those performing PVN ICSS. These differences in response patterns may indicate differences in the underlying mechanisms of the reinforcing effects of PVN and MFB ICSS; however these differences could also be explained if the reinforcing effects of PVN stimulation are mediated by unintended (and weak) stimulation of the MFB. It is noteworthy that stimulation of the MFB did not impact mechanical allodynia however. This indicates not only that stimulation of limbic dopamine does not alter spinally-mediated hypersensitivity, but that stimulation of the MFB unlikely stimulated the PVN; it is unclear if the reverse is true, however.

To further explore the mechanisms mediating PVN ICSS, as well as to distinguish these from MFB ICSS, pharmacological experiments targeting oxytocin and opioid receptors were performed during ICSS sessions. Intrathecal atosiban significantly decreased responding for PVN ICSS in SNL rats, which resulted in rightward shifts in the frequency response curve. Interestingly, spinal morphine and naltrexone did not affect PVN ICSS in SNL rats. This suggests that spinal opioid release does not mediate the reinforcing effects of PVN ICSS in SNL rats, which is similar to the effects on mechanical allodynia. Systemic administration of atosiban and naltrexone did not alter PVN ICSS in SNL rats, which is also consistent with their lack off effect on reversal of hypersensitivity.
with PVN stimulation. Conversely, spinal atosiban produced a less consistent effect in decreasing responding for PVN ICSS in control rats; spinal atosiban was also without effect in control and SNL rats responding for MFB ICSS, thus indicating selectivity of effect for PVN ICSS in SNL rats. Collectively, these results implicate spinal oxytocin release in partially mediating the anti-allodynic and reinforcing effects of PVN ICSS in SNL rats.

Selective effects of spinal atosiban suggests mechanistic differences mediating PVN and MFB ICSS, yet does not explain why PVN ICSS is reinforcing in control rats. One possibility is that PVN stimulation indirectly stimulates mesolimbic dopamine. The mesolimbic dopamine system arises from dopamine cell bodies in the ventral tegmental area (VTA) which project to the nucleus accumbens (NAcc), a pathway implicated in the reinforcing aspects of rewarding stimuli. PVN oxytocin fibers project to the VTA, and direct injection of oxytocin into the VTA causes dopamine release in the NAcc. It is likely that electrical stimulation of the PVN releases oxytocin in the VTA, which may be reinforcing in normal and chronic pain states. It is unclear if relevant amounts of atosiban reached the brain following intrathecal injection; if so the inhibitory effects of atosiban on PVN ICSS may reflect inhibition of oxytocin receptors in the limbic system. Future studies could address this question through the use of ICV or direct injections into discrete brain regions.

It is worth noting that compared to previous findings, higher frequencies of PVN stimulation were necessary to reverse hypersensitivity following nerve injury. Initial attempts to provide short, constant stimulation followed by
assessment of allodynia proved difficult to do in freely moving rats. Therefore, in
the current study PVN stimulation was delivered for minutes, and each 0.5-sec
train of stimulation was followed by a 1.5-sec period of no stimulation. This was
done because it allowed animals to habituate to the stimulation, reduced motor
effects, allowed testing at higher frequencies, and modeled rates of self-
stimulation during ICSS sessions. It is quite possible that the repeated use of on
and off stimulation may have led to the higher frequency requirements in
reversing hypersensitivity. Nonetheless, this protocol was reliable in reversing
hypersensitivity that was in turn sensitive to pharmacological manipulations.

One limitation of ICSS is the lack of selectivity of electricity. Although
anatomically the oxytocinergic system supports a role for analgesic and
reinforcing effects associated with PVN stimulation, non-oxytocinergic neurons
were undoubtedly stimulated and may contribute to the analgesic and reinforcing
effects of PVN stimulation. ICSS is also further complicated when studying brain
regions located in close proximity to one another, such as the PVN and MFB,
necessitating the use of other manipulations (e.g., pharmacological) to
distinguish stimulation effects. Under such circumstances an optogenetic
approach would be ideal. Another limitation regarding selectivity is the use of
atosiban, which antagonizes oxytocin and vasopressin receptors. Oxytocin and
vasopressin are relatively promiscuous in binding to both oxytocin and
vasopressin receptors, therefore it is possible that the effects of PVN
stimulation are mediated by oxytocin and/or vasopressin release. Further, these
effects may be mediated through oxytocin receptors, vasopressin receptors, or
both; future studies with selective antagonists would be helpful in verifying the
relative contributions of both in these effects.

In conclusion, electrical stimulation of the PVN alleviated mechanical allodynia
and produced reinforcing effects at similar frequencies in SNL rats. Both of these
effects were diminished by intrathecal atosiban, but not naltrexone, suggesting a
role of oxytocin but not opioid release in mediating these effects. Surprisingly,
control rats lever pressed for PVN ICSS at similar rates and frequencies to SNL
rats, suggesting that PVN stimulation produces effects other than pain relief that
are reinforcing in non pain states. However, intrathecal atosiban did not
significantly alter PVN ICSS in control rats, suggesting that spinal oxytocin
influences the reinforcing effects of PVN ICSS to a greater degree under chronic
pain states. In addition, intrathecal atosiban was without effect in control and SNL
rats performing MFB ICSS, indicating specificity of effect for PVN stimulation.
Further, systemic administration of atosiban and naltrexone did not effect PVN or
MFB ICSS in control and SNL rats. Collectively, these findings suggest that
central release of oxytocin produces reinforcing effects during chronic pain
states, which may be mediated through interactions with limbic regions
implicated in reward and analgesia.
Acknowledgements.

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland.
References

1. Mogil JS, Crager SE: What should we be measuring in behavioral studies of chronic pain in animals? Pain 2004; 112: 12-5


13. Ewan EE, Martin TJ: Opioid facilitation of rewarding electrical brain stimulation is suppressed in rats with neuropathic pain. Anesthesiology 2011; 114: 624-32


CHAPTER VI

INTRACRANIAL SELF-STIMULATION OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS: INCREASED FACILITATION BY MORPHINE COMPARED TO COCAINE

Eric E. Ewan and Thomas J. Martin

The following chapter is being submitted to *Anesthesiology*, and is reprinted with permission. Stylistic variations are due to conforming to the publishing journal. Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design and manuscript preparation.
Abstract

**Introduction.** Neuropathic pain attenuates the ability of opioids to facilitate rewarding electrical stimulation of limbic dopaminergic pathways originating from the ventral tegmental area. We have recently demonstrated that electrical stimulation of the paraventricular nucleus (PVN) of the hypothalamus is reinforcing in rats, however the effects of opioids on this system and the influence of neuropathic pain is unknown.

**Methods.** Control and spinal nerve ligated (SNL) rats were implanted with electrodes into the PVN or medial forebrain bundle (MFB). Control and SNL rats were trained to lever press for electrical stimulation of the PVN or MFB (ICSS), and modulation by morphine or cocaine was assessed.

**Results.** Control and SNL rats lever pressed for stimulation of the PVN and MFB. Morphine produced greater reductions in the frequency at which rats emitted 50% of maximal responding (EF50) for PVN ICSS (maximal effect 24.67 ± 4.60 and 24.11 ± 5.96 in SNL and control rats, respectively) compared to MFB ICSS (12.38 ± 6.77 and 10.90 ± 3.25). In contrast, cocaine was less efficacious in potentiating PVN ICSS (maximal effect 11.76 ± 2.86 and 12.38 ± 4.01 in SNL and control rats, respectively) compared to MFB ICSS (30.58 ± 3.40 and 27.55 ± 4.51).

**Conclusions.** PVN ICSS is facilitated to a greater extent by morphine than cocaine and the effects of neither drug on this behavior are altered by SNL. The PVN appears to have a greater role in the reinforcing effects of opioids than classical limbic regions, particularly in the presence of chronic pain.
Introduction

Intracranial self-stimulation (ICSS) is an operant paradigm pairing lever presses with electrical stimulation of discrete brain pathways.\textsuperscript{1} For decades it has been known that rats will lever press for stimulation of brain regions within the mesolimbic dopamine system, including the ventral tegmental area (VTA) and medial forebrain bundle (MFB).\textsuperscript{2} Drugs of abuse such as opioids and cocaine, whose reinforcing effects are mediated through this circuitry, potentiate the reinforcing effects of VTA and MFB ICSS in rats.\textsuperscript{2} Although neuropathic pain does not alter VTA ICSS, the ability of morphine, heroin, and commonly abused prescription opioids are less effective in facilitating VTA ICSS in SNL rats compared to control animals.\textsuperscript{3,4} It is not known if this effect of SNL is limited to facilitation of ICSS in the VTA. We have recently shown that electrical stimulation of the hypothalamic paraventricular nucleus (PVN) produces reinforcing effects in normal and nerve-injured rats (Ewan & Martin, this issue), effects which may be mediated by oxytocin release in the limbic system.\textsuperscript{5}

Oxytocin release is stimulated in response to a number of environmental stimuli and is involved in a host of behavioral effects including maternal and social bonding, anxiety, and pain among others.\textsuperscript{6} A lesser understood effect of oxytocin is its ability to modulate behavioral effects of drugs of abuse.\textsuperscript{7} These effects likely arise from release of oxytocin from the PVN into limbic and forebrain regions, where oxytocin may interact with dopamine neurotransmission to modulate aspects of drug addiction.\textsuperscript{5} For instance, PVN oxytocin fibers project to the VTA,\textsuperscript{8} which expresses oxytocin receptor mRNA,\textsuperscript{9} and intra-VTA oxytocin
stimulates dopamine release in the NAcc that is reversed by pretreatment with an intra-VTA oxytocin receptor antagonist. Intracerebroventricular administration of oxytocin has been shown to inhibit analgesic tolerance to heroin and morphine. In addition, intravenous self-administration of heroin is reduced following intra-accumbens or intra-hippocampal oxytocin administration in rats, though it is unclear if this reflects changes in tolerance or in the rewarding properties of heroin. Oxytocin administered intracerebroventricularly has also been shown to inhibit some of the acute behavioral effects of cocaine, including stereotypy. It is therefore clear that oxytocin has the potential to modulate effects of drugs of abuse, though it is unclear to what extent the reverse is true.

It is hypothesized that the reinforcing effects of PVN ICSS in normal and nerve-injured animals (Ewan & Martin, this issue) are mediated to some extent through indirect stimulation of mesolimbic dopamine via oxytocin release. It would be expected that drugs of abuse, which stimulate mesolimbic dopaminergic neurons, would subsequently enhance the reinforcing effects of PVN ICSS. The current study sought to address this by assessing the effects of morphine and cocaine on the reinforcing effects of PVN ICSS. Given the close proximity of the PVN to the MFB, the modulatory effects of morphine and cocaine on the reinforcing effects of MFB ICSS were also assessed. It is also unclear to what extent nerve injury, which diminishes the potentiating effects of opioids for VTA ICSS, will alter the ability of morphine and cocaine to facilitate PVN and MFB ICSS. Therefore, studies were performed in both normal and nerve-injured rats.
Materials and Methods

Subjects

Subjects were 39 male, Fisher 344 rats (12 SNL and 11 control rats for PVN experiments; 9 SNL and 7 control rats for MFB experiments) weighing between 275-325 g at the beginning of the experiment (Harlan Laboratories, Raleigh, NC). Rats were group-housed in a temperature and humidity controlled room that was maintained on a reversed light-dark cycle (dark 05:00-17:00); this room was adjacent to the room in which behavioral experiments were performed. Food and water were continuously available except during behavioral experiments. All procedures were conducted according to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina. The subjects used for these studies were the same animals used for previously presented experiments on PVN and MFB ICSS (Ewan and Martin, this issue).

Surgeries

Electrode Implantation. Rats were permanently implanted with electrodes as previously described. Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal) and received penicillin G procaine (75,000 U, intramuscular) to prevent infection. Rats were secured in a stereotaxic frame and platinum bipolar stimulating electrodes (Plastics One, Roanoke, VA) were aimed at the left PVN or left MFB (2.5mm and 3.0mm posterior to bregma, 0.3mm and 1.7mm lateral from the midline, and
8.0mm and 8.5mm below the skull, for PVN and MFB, respectively), which were secured by three stainless steel screws embedded in dental acrylic on the skull surface.

**Spinal Nerve Ligation.** After electrode implantation 21 of the 39 rats were subjected to SNL as previously described. Briefly, an incision was made through the skin and muscle of the lower back, and the left transverse process of the fifth lumbar vertebra was removed using bone microrongeurs. The fifth lumbar nerve was exteriorized and ligated with 4.0 silk suture. The sixth lumbar nerve was exteriorized from below the iliac bone at the sciatic notch and similarly ligated. Afterwards, muscle layers were sutured with 4.0 chromic gut, the skin with 4.0 nylon suture, and exterior wounds dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

**Paw Withdrawal Threshold**

Mechanical allodynia was assessed using von Frey filaments (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL) for all animals using Dixon non-parametric statistics. PWT’s were assessed 14 days after surgery to verify development of allodynia (PWT<4.0 g).

**Drugs**

Morphine sulfate was purchased as a 15 mg/ml sterile solution (Baxter Healthcare; Deerfield, IL). Cocaine hydrochloride was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD), dissolved in 0.9% (wt/vol) saline, and sterilized by filtration through a 0.22 μm nitrocellulose filter. All drugs were diluted using 0.9% (wt/vol) saline, pH 7.4.
Intracranial Self-Stimulation (ICSS)

**Apparatus.** Operant chambers housed within sound- and light-attenuating enclosures equipped with a houselight and ventilation fan were used (Med Associates Inc., St. Albans, VT). These chambers have a lever 5 cm above a grid bar floor, stimulus lamp 2 cm above the lever, and a tone generator. An ICSS stimulator controlled by computer software (Med Associates Inc.) that controlled all stimulation parameters and data collection was located outside of the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One) positioned above the operant chamber connected the electrodes to the ICSS stimulator via 25cm cables (Plastics One).

**Behavioral Procedure.** Following at least 14 days recovery from surgery, rats were trained to lever press for electrical stimulation of the PVN or MFB as previously described. Illumination of the stimulus light indicated stimulus availability and each lever press generated a 0.5-sec train of rectangular alternating cathodal and anodal pulses (0.1-ms pulse durations). During stimulation the stimulus light shut off, the houselight turned on, and a tone sounded. Responding during stimulation resulted in no further stimulation and was not recorded.

During training sessions the frequency was held constant (150Hz) and the intensity adjusted to maintain consistent responding. Following initial training, frequency response curves were generated. For PVN ICSS, 120-min sessions consisted of five 18-min components. Each component consisted of eight 135-sec trials. Each trial began with a 5-sec timeout followed by a 10-sec priming
period in which rats received five noncontingent stimulations, and concluded with a 120-sec response period. Current intensity remained constant (unique to each animal) and 8 frequencies (158-71 Hz, 0.05 log increments; PVN) corresponding to each trial were made available in descending order. A 30-min timeout between the third and fourth components permitted drug injections. MFB ICSS was similarly performed with the following differences: 90-min sessions consisted of six 10-min components, which consisted of ten 60-sec trials. Each trial began with a 5-sec timeout, followed by a 5-sec priming period, and concluded with a 50-sec response period; 10 frequencies (156-45 Hz, 0.06 log increments) corresponded to each trial. At the beginning of the timeout rats received 1 mL/kg intraperitoneal injections of saline (0.9% wt/vol), morphine (0.3 - 6mg/kg), or cocaine (5 - 10 mg/kg). For data analysis, the 2 components preceding drug injection and the 2 (PVN) or 3 (MFB) components following drug injection were averaged and compared using Prism software (sigmoidal-dose response, variable slope; Graph Pad, La Jolla, CA). Test sessions were separated by at least 1 day. Test sessions were performed 1-4 months after initial surgery.

Histology

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed, frozen in isopentane (-35°C), and stored at -80°C. Coronal sections (25µm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the PVN and MFB (Figure 1).
Figure 1. Location of the stimulating electrodes within the paraventricular nucleus of the hypothalamus and medial forebrain bundle for control and spinal nerve-ligated (SNL) rats. Numbers left of each brain section indicate distance posterior to bregma, according to the atlas of Paxinos and Watson (1998).21
Data Analysis

The EF50 (frequency at which rats emitted 50% of maximal responding) and maximum response rate for PVN or MFB ICSS was calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad). The effect of drug treatment on PVN and MFB ICSS was analyzed using a one-way ANOVA with drug dose serving as the independent variables and \( \Delta \text{EF50} \) (EF50 prior to injection – EF50 after injection) or maximal response rates serving as the dependent measures. Post-hoc analyses within the control or SNL groups were made using Dunnett’s t-test for multiple comparisons with saline injection serving as control. A two-tailed p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc., Cary, NC).
Results

Effects of SNL on ICSS of the PVN or MFB.

The intensities used for ICSS were not significantly different between SNL and control groups for either the PVN [F(1,22)=0.2, p=0.6] or the MFB [F(1,15)=0.05, p=0.8]. The maximum response rates also did not differ between control and SNL rats for ICSS in the PVN [F(1,22)=0.2, p=0.6] or in the MFB [F(1,15)=3.7, p=0.07] (Figure 2). The EF50 values did not differ between SNL and control rats for ICSS in the MFB [F(1,15)=0.2, p=0.7], however the EF50 value for ICSS in the PVN was slightly but significantly greater in SNL rats (114.7±1.8) compared to control subjects (109.1±1.8) [F(1,22)=4.6, p=0.04]. These data have been reported for these animals elsewhere (Ewan and Martin, this issue).

Comparison of ICSS parameters in the PVN and MFB.

The intensities used for ICSS did not differ (SNL and control rats combined) between the PVN and MFB [F(1,38)=2.1, p=0.16]. The maximum response rate maintained by ICSS in the PVN (23.8±1.1 resp/min) was approximately half that maintained in the MFB (45.1±1.3 resp/min) [F(1,38)=160, p<0.0001] (Figure 2). The EF50 was also significantly greater for ICSS in the PVN (112.0±1.6) compared to the MFB (96.0±2.0) [F(1,38)=39, p<0.0001]. These data have been reported for these animals elsewhere (Ewan and Martin, this issue).

Effects of morphine on PVN ICSS in control and SNL rats.

In control rats, intraperitoneal administration of saline had no effect on the EF50 or Rmax for PVN ICSS. Morphine produced a dose-dependent leftward
Figure 2. Baseline responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. Frequency-response curves for baseline responding were generated by averaging the second and third components preceding saline administration (prior to any drug treatments). The y-axis indicates the number of self-stimulations (0.5-sec) per 60 seconds for each frequency (x-axis). Data shown are averages across control (n=11, n=7) and SNL (n=12, n=9) rats with PVN and MFB electrodes, respectively. Average current intensities during baseline responding were 246.82 (17.51) uA and 215.71 (26.87) uA for control and 234.58 (19.59) uA and 208.33 (18.86) uA for SNL rats with PVN and MFB electrodes, respectively.
shift in the frequency-rate curves for PVN ICSS \([F(4,45)=7.6, \ p<0.0001]\), producing significant decreases in the EF50 at doses of 3 and 6 mg/kg compared to saline (Figure 3A). The Rmax value was also significantly decreased following administration of 3 mg/kg of morphine \((p \leq 0.05)\). In SNL rats, morphine produced a significant leftward shift in the frequency-rate curves for ICSS of the PVN \([F(4,39)=14.3, \ p<0.0001]\) with doses of 1, 3, and 6 mg/kg producing decreases in the EF50 values greater than that of saline \((p \leq 0.05)\) (Figure 3A). The 3 mg/kg dose of morphine significantly decreased the Rmax value for PVN ICSS in SNL rats as well \((p \leq 0.05)\).

**Effects of morphine on MFB ICSS in control and SNL rats.**

In control rats, intraperitoneal administration of saline had no effect on either the EF50 or Rmax for MFB ICSS. Intraperitoneal administration of morphine significantly shifted the rate-frequency curve to the left in a dose-dependent manner \([F(3,26)=6.0, \ p=0.004]\) while having no effect on the Rmax \([F(3,26)=0.8, \ p=0.5]\). All doses greater than 0.3 mg/kg of morphine produced a significant shift in the EF50 compared to saline \((p \leq 0.05)\). (Figure 3A). Conversely, in SNL rats none of the doses tested of morphine produced a leftward shift in the rate-frequency curves for MFB ICSS that was significantly different from saline and none altered the Rmax values \((p \leq 0.05)\) (Figure 3A).

**Effects of cocaine on PVN ICSS in control and SNL rats**

Cocaine produced a significant leftward shift in the frequency-rate curve for ICSS of the PVN in control rats \([F(2,25)=3.8, \ p=0.04]\) with only the highest dose of 10 mg/kg producing a significant decrease in the EF50 value compared to
Figure 3. Effects of morphine on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the two (PVN) and three (MFB) components following drug injection from the EF50 for the two components preceding drug injection. Data shown are averages across control (n=8, except n=11 for 3mg/kg) and SNL (n=7, except n=8 and n=6 for 3 and 6mg/kg, respectively) rats with PVN electrodes, as well as control (n=7), and SNL (n=8) rats with MFB electrodes. Frequency-response curves before and after 6mg/kg morphine (30-min) are shown for SNL rats with PVN (B, n=6) and MFB (C, n=8) electrodes. * Significantly different from saline treatment P ≤ 0.05.
saline treatment ($p \leq 0.05$) (Figure 4A). Neither dose of cocaine significantly altered the Rmax values for ICSS of the PVN. The effects of cocaine on ICSS of the PVN was similar between SNL and control subjects, producing a leftward shift in the frequency rate curve [$F(2, 32)=4.7, p=0.02$] that was significant only at the highest dose tested (10 mg/kg, $p \leq 0.05$) (Figure 4A). Cocaine did not produce any effects on Rmax values in SNL rats [$F(2,32)=1.2, p=0.3$].

**Effects of cocaine on MFB ICSS in control and SNL rats.**

Cocaine also produced a dose-dependent leftward shift in the rate frequency curve for MFB ICSS [$F(2,20)=14.8, p<0.0001$] and increased the Rmax as well [$F(2,20)=18.8, p<0.0001$], with both the 5 and 10 mg/kg doses producing a greater effect than saline on both parameters ($p \leq 0.005$) (Figure 4A). In SNL rats, intraperitoneal administration of saline also did not alter either the EF50 or the Rmax for MFB ICSS. Cocaine produced a dose-dependent leftward shift in MFB ICSS in SNL rats [$F(2,25)=32.7, p<0.0001$] and increased Rmax values as well [$F(2,25)=12.3, p<0.0001$] (Figure 4A). As in control subjects, both doses of cocaine significantly decreased the EF50 values and increased the Rmax values.
Figure 4. Effects of cocaine on responding for electrical stimulation of the paraventricular nucleus of the hypothalamus (PVN) and medial forebrain bundle (MFB) for control and spinal nerve-ligated (SNL) rats. (A) Reduction in EF50 (frequency at which rats emitted 50% of maximal responding) was calculated by subtracting the EF50 for the two (PVN) and three (MFB) components following drug injection from the EF50 for the two components preceding drug injection. Data shown are averages across control (n=8 and n=7 for 5 and 10mg/kg, respectively) and SNL (n=9 and n=12 for 5 and 10mg/kg, respectively) rats with PVN electrodes, as well as control (n=7), and SNL (n=8 and n=9 for 5 and 10mg/kg, respectively) rats with MFB electrodes. Frequency-response curves before and after 5mg/kg morphine (30-min) are shown for SNL rats with PVN (B, n=9) and MFB (C, n=8) electrodes. * Significantly different from saline treatment P ≤ 0.05.
Discussion

The current study sought to further elucidate the potential mechanisms underlying the reinforcing effects of PVN ICSS, as well as to determine if they are modulated by drugs of abuse in a manner reported previously for the VTA. Pharmacology studies revealed that morphine and cocaine potentiated the reinforcing effects of PVN and MFB ICSS, though important differences in drug efficacy for each brain region were observed. Morphine was more efficacious in potentiating the reinforcing effects of PVN compared to MFB ICSS. In contrast, cocaine had decreased potency and efficacy in potentiating PVN compared to MFB ICSS. Studies in SNL rats revealed that nerve injury diminished morphine’s ability to facilitate MFB ICSS, similar to previous results with VTA ICSS, but did not diminish morphine’s ability to facilitate PVN ICSS. Cocaine’s effects were unchanged by SNL for either PVN or MFB ICSS, similar to previous findings with VTA ICSS. These studies suggest that the reinforcing effects of PVN ICSS may be modulated to some extent through indirect stimulation of mesolimbic dopamine pathways however it is likely that other systems are involved as well, since PVN ICSS is influenced to a greater extent by opioids compared to cocaine while the reverse is true with MFB or VTA ICSS.

It is hypothesized that PVN stimulation is reinforcing to some extent through indirect stimulation of mesolimbic dopamine neurons of the VTA, a pathway implicated in the reinforcing aspects of rewarding stimuli. PVN oxytocin fibers project directly to the VTA and terminate in close proximity to VTA dopamine cell bodies, providing a neuroanatomical basis for oxytocin interactions with
mesolimbic dopamine circuitry. Therefore, PVN stimulation is expected to induce oxytocin release in the VTA. Exogenous administration of oxytocin in the VTA induces dopamine release in the NAcc,⁸ and thus PVN stimulation-induced oxytocin release in this region would similarly be expected to stimulate dopamine neurotransmission. Under these conditions, it would be expected that drugs which stimulate this circuitry would facilitate PVN ICSS, and the fact that morphine and cocaine facilitated PVN ICSS is in agreement for a role of mesolimbic dopamine mediating the reinforcing effects of PVN ICSS.

Opioids and dopamine agonists also potentiate the effects of MFB ICSS.² Given the close proximity of the PVN to MFB, one possibility is that unintended stimulation of the MFB is responsible for the reinforcing effects of PVN ICSS, and moreover may explain why morphine and cocaine facilitated PVN ICSS. The potency and efficacy of cocaine was diminished in rats responding for PVN compared to MFB ICSS in both control and SNL rats. If PVN ICSS were mediated by unintended stimulation of the MFB, then these results would indicate that weak stimulation of the MFB from a distal electrode diminishes the potency and efficacy of a drug to facilitate ICSS. However, such an interpretation is unlikely since in the same rats morphine produced greater facilitation of PVN compared to MFB ICSS. One of the most striking of the differences observed between PVN and MFB ICSS was the relative inability of cocaine to facilitate PVN ICSS compared to the MFB. For MFB ICSS the highest dose of cocaine assessed produced roughly a 2.5 fold greater facilitation compared to morphine, similar to previous findings using VTA ICSS,³ and consistent with microdialysis
data indicating that cocaine stimulates dopamine release in the NAcc to a greater extent than morphine.\textsuperscript{18} In contrast, this same dose of cocaine was only half as efficacious as morphine in facilitating PVN ICSS in control and SNL rats. This suggests a greater role for opioid modulation of PVN ICSS compared to MFB ICSS, and further supports the notion that the neuronal mechanisms that support PVN ICSS are fundamentally different than those involved in MFB ICSS.

In the current study all manipulations on ICSS were performed in both control and SNL rats. The only effect of nerve injury on altering drug modulation of PVN and MFB ICSS was to decrease the potency of morphine for potentiation of MFB ICSS. This finding is similar to previous work using VTA ICSS,\textsuperscript{3} and may relate to impairment of mu opioid receptor function within the VTA.\textsuperscript{19} The working hypothesis is that opioids are less effective in stimulating the mesolimbic dopaminergic system in the presence of neuropathic pain,\textsuperscript{19} and therefore are less effective in facilitating either VTA or MFB ICSS in nerve-injured rats. The fact that morphine was equally effective at facilitating PVN ICSS in SNL and control rats is yet another key difference between PVN and MFB ICSS, and indicates that PVN ICSS may not solely be mediated by indirect stimulation of mesolimbic dopaminergic systems, since one would expect morphine to be less efficacious in SNL rats compared to control subjects if this was true. It is possible that morphine facilitates PVN ICSS by stimulating oxytocin release and/or interacting with oxytocin neurotransmission throughout the limbic system, as well as through its own direct actions in stimulating dopamine neurotransmission.\textsuperscript{20} Such additive effects could also explain why morphine produces greater
facilitation of PVN compared to MFB ICSS, and may mask the suppressive effects of SNL on opioid activity within the VTA. Similarly, it is possible that the reduced efficacy of cocaine compared to morphine in potentiating PVN ICSS may be due to cocaine exerting direct effects on limbic dopamine neurotransmission, but having little impact on facilitating oxytocin release and/or interacting at other sites that contribute to the reinforcing effects of PVN ICSS. To this end, increased efficacy of morphine compared to cocaine in facilitating PVN ICSS suggests that opioid interactions with the PVN may play a prominent and relatively unknown role in the reinforcing and abuse related effects of opioids, during both pain and non-pain states.

As has been stated elsewhere (Ewan & Martin, this issue) a serious limitation of the ICSS methodology is the lack of selectivity of electrical stimulation, particularly when studying brain regions in close proximity to each other. To combat this, pharmacological studies were performed in groups with electrodes placed in the PVN and MFB, in order to differentiate the modulatory effects of morphine and cocaine. Since morphine and cocaine facilitate PVN and MFB ICSS, albeit to different degrees, it is impossible to rule out the existence of unintended stimulation reaching the opposing brain region. It should be noted, however, that the differential effects of morphine and cocaine in facilitating PVN and MFB ICSS were obtained in both control and SNL rats; this increases confidence in these findings since in all cases critical pharmacological differences were found in two groups of animals for each brain region.
Another limitation of the current work pertains to regional selectivity of the drugs studied. Since morphine and cocaine were given systemically, it is unclear to what degree drug effects are attributable to specific brain regions. This is particularly important when trying to understand the mechanism by which morphine facilitates PVN ICSS, since under the current thinking PVN ICSS could be modulated at the level of the PVN, VTA, or in other limbic and frontal brain regions that contain opioid receptors. Future studies addressing these questions would benefit from the use of site specific intracranial injections of opioid and oxytocin agonists and antagonists.

In conclusion, electrical stimulation of the PVN produces reinforcing effects in control and SNL rats. It is hypothesized that oxytocin release into limbic regions involved in reward (e.g., VTA) likely mediates to some degree the reinforcing effects of PVN ICSS. Morphine was more effective than cocaine in potentiating PVN ICSS, while the reverse is true in the MFB. The reinforcing effects of PVN ICSS are influenced by opioids to a greater extent than cocaine and these effects are unaltered by SNL, suggesting that the PVN may be a brain region intricately involved in the reinforcing effects and abuse liability of opioids under normal as well as chronic pain states.
Acknowledgements.

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland.
References


3. Ewan EE, Martin TJ: Opioid facilitation of rewarding electrical brain stimulation is suppressed in rats with neuropathic pain. Anesthesiology 2011; 114: 624-32


5. Baskerville TA, Douglas AJ: Dopamine and oxytocin interactions underlying behaviors: potential contributions to behavioral disorders. CNS Neurosci Ther 2010; 16: e92-123


CHAPTER VII

ELECTRICAL STIMULATION OF THE PERIAQUEDUCTAL GRAY FAILS TO ELICIT OPERANT BEHAVIOR IN NERVE-INJURED RATS

Eric E. Ewan and Thomas J. Martin

Eric E. Ewan performed the experimental work and Thomas J. Martin advised the experimental design.
Abstract

Deep brain stimulation of the periaqueductal gray (PAG) has proven efficacious in some instances in the treatment of pain that is poorly managed by pharmacotherapies. The PAG plays a prominent role in descending pain modulation, and electrical stimulation of the PAG produces analgesia in preclinical models assessing evoked pain (e.g., hypersensitivity). In the current study rats were subjected to spinal nerve ligation (SNL) and implanted with electrodes into the left ventral PAG. Electrical stimulation of the ventral PAG (50 and 100Hz) produced a significant increase in paw withdrawal threshold (maximum threshold 11.7 ± 3.0 g) compared to baseline (2.64 ± 0.3). Attempts were made to train lever pressing for intracranial self-stimulation (ICSS). After 10 days of training, none of the 8 SNL rats lever pressed for PAG stimulation. These experiments indicate that stimulation of the ventral PAG alleviates mechanical hypersensitivity but does not produce reinforcing effects after nerve injury, suggesting that relief of allodynia per se does not produce reinforcing effects in SNL rats.
Introduction

Deep brain stimulation has been used to treat drug-resistant pain. Beneficial effects were first reported with stimulation of the thalamus and periaqueductal gray (PAG) (Gybels & Kupers, 1990), and more recent success has been reported with motor cortex stimulation (Carroll et al., 2000). Animal studies investigating DBS are limited, with most experiments assessing evoked pain (e.g., hypersensitivity) in normal or nerve-injured rats. The primary complaint of patients with neuropathies is spontaneous pain, (Backonja & Stacey, 2004) which is difficult to assess in animals. Interestingly, motor cortex stimulation produces conditioned place preference (CPP) selectively in rats with spinal cord lesions (Davoody et al., 2011), suggesting that stimulation of brain regions involved in relief of ongoing pain may be reinforcing during chronic pain states in rats.

Intracranial self-stimulation (ICSS) is one technique capable of assessing the reinforcing effects of brain stimulation. During ICSS sessions rats can lever press to electrically stimulate a discrete brain region through a surgically implanted electrode. Rats will lever press for stimulation of brain regions implicated in positive reinforcement, such as the ventral tegmental area and medial forebrain bundle, which stimulates dopamine release in the nucleus accumbens. It is therefore hypothesized that stimulation of brain regions which alleviate ongoing pain following nerve injury should produce negative reinforcement, and similarly maintain operant behavior in nerve-injured rats.
The most extensively studied brain region with DBS in animals is the PAG. It was initially shown that rats could endure painful laparotomies without anesthesia during stimulation of the PAG (Reynolds, 1969). Further investigations have demonstrated that dorsal PAG stimulation is aversive (Oliveras & Besson, 1988), while ventral PAG stimulation is analgesic (Fardin, Oliveras, & Besson, 1984) in rats. PAG stimulation-induced analgesia likely is mediated by descending projections to the RVM (Basbaum & Fields, 1984) and may be opioid mediated, since the antiallodynic effects of PAG stimulation are reversed by systemic naloxone (Lee, Park, Won, Park, & Sohn, 2000).

The current study sought to determine if ventral PAG stimulation produces analgesia, and if this in turn produces reinforcing effects in nerve-injured rats. Therefore, the ability of ventral PAG stimulation to alleviate mechanical allodynia following nerve injury was assessed. In addition, the reinforcing effects of ventral PAG stimulation were assessed using the ICSS paradigm.
Materials and Methods

Subjects

Subjects were 8 male, Fisher 344 rats weighing between 275-325 g at the beginning of the experiment (Harlan Laboratories, Raleigh, NC). Rats were group-housed in a temperature and humidity controlled room that was maintained on a reversed light-dark cycle (dark 05:00-17:00); this room was adjacent to the room in which behavioral experiments were performed. Food and water were continuously available except during behavioral experiments. All procedures were conducted according to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Surgeries

Electrode Implantation. Rats were permanently implanted with electrodes as previously described.(Ewan & Martin, 2011) Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and atropine methyl nitrate (10mg/kg, intraperitoneal) and received penicillin G procaine (75,000 U, intramuscular) to prevent infection. Rats were secured in a stereotaxic frame and platinum bipolar stimulating electrodes (Plastics One, Roanoke, VA) were aimed at the left PAG (coordinates?), which were secured by three stainless steel screws embedded in dental acrylic on the skull surface.

Spinal Nerve Ligation. After electrode implantation rats were subjected to SNL as previously described.(Martin, Kim, Buechler, Porreca, & Eisenach, 2007)
Briefly, an incision was made through the skin and muscle of the lower back, and the left transverse process of the fifth lumbar vertebra was removed using bone microrongeurs. The fifth lumbar nerve was exteriorized and ligated with 4.0 silk suture. The sixth lumbar nerve was exteriorized from below the iliac bone at the sciatic notch and similarly ligated. Afterwards, muscle layers were sutured with 4.0 chromic gut, the skin with 4.0 nylon suture, and exterior wounds dressed with antibiotic powder (Polysporin; Pfizer Healthcare, Morris Plains, NJ).

**Paw Withdrawal Threshold**

Mechanical allodynia was assessed using von Frey filaments (Touch Test Sensory Evaluators; Stoelting, Wood Dale, IL)(Nichols, Bian, Ossipov, Lai, & Porreca, 1995) for all animals using Dixon non-parametric statistics.(Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994) PWT’s were assessed 14 days after surgery to verify development of allodynia. Experimenter-delivered electrical stimulation (40 0.5sec stimulations per minute) of the ventral PAG was delivered at the highest current intensity (uA) that did not produce motor abnormalities (unique for each rat) during testing. Baseline PWT’s were determined immediately before stimulation. Stimulation was delivered for 60-sec prior to assessment at each frequency. PWT’s were assessed following stimulation at 10, 50 and 100 Hz in ascending order, with 15-min of non-stimulation between test periods. Testing lasted between 1-2 minutes for each frequency.

**Intracranial Self-Stimulation (ICSS)**

*Apparatus.* Operant chambers housed within sound- and light-attenuating enclosures equipped with a houselight and ventilation fan were used (Med
Associates Inc., St. Albans, VT). These chambers have a lever 5 cm above a
grid bar floor, stimulus lamp 2 cm above the lever, and a tone generator. An
ICSS stimulator controlled by computer software (Med Associates Inc.) that
controlled all stimulation parameters and data collection was located outside of
the enclosure. A 2-channel swivel commutator (Model SLC2C, Plastics One)
positioned above the operant chamber connected the electrodes to the ICSS
stimulator via 25cm cables (Plastics One).

**Behavioral Procedure.** Following at least 14 days recovery from surgery, rats
were trained to lever press for brain stimulation of the PAG as previously
described (Ewan & Martin, 2011). Illumination of the stimulus light indicated
stimulus availability and each lever press generated a 0.5-sec train of rectangular
alternating cathodal and anodal pulses (0.1-ms pulse durations). During
stimulation the stimulus light shut off, the houselight turned on, and a tone
sounded. Responding during stimulation resulted in no further stimulation and
was not recorded.

During training sessions the frequency was initially set to 100Hz and the
intensity adjusted in an attempt to establish and maintain consistent responding.
Training involved shaping rats’ behavior by experimentally delivering stimulation
for successive approximations to the target behavior (lever pressing); these
include stimulation for orienting toward, approaching, and sniffing the lever. If
stimulation produced no overt behavioral effects and did not increase the
occurrence of the successive approximations, the intensity was increased in
50uA increments, up to 250 uA. Similarly, if stimulation elicited aversive or
avoidance-like behaviors, intensity was decreased. If after 3 days of training minimal or no responding had occurred, the highest intensity (uA) that didn’t produce aversive or avoidance-like behaviors was set constant, and frequencies varying from 25 to 150 Hz were delivered during training sessions using similar shaping procedures as described. Training sessions were discontinued after 10 days if responding was not established.

Histology

Rats were sacrificed by carbon dioxide asphyxiation. Brains were rapidly removed, frozen in isopentane (-35°C), and stored at -80°C. Coronal sections (25μm) around the electrode tract were obtained using a cryostat to confirm electrode placement within the ventral PAG. (Figure 1)

Data Analysis

Data for PWT’s was analyzed using a one-way ANOVA with frequency serving as the independent variable. A two-tailed p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc., Cary, NC).
Figure 1. Location of the stimulating electrodes within the periaqueductal gray in spinal nerve-ligated (SNL) rats. Numbers left of each brain section indicate distance posterior to bregma, according to the atlas of Paxinos and Watson (1998).
Results

Anti-allodynic effects of PAG stimulation.

Electrical stimulation of the PAG reversed mechanical allodynia in SNL rats in a frequency dependent manner \[F(3,27)=6.1, \ p=0.003]\], with PWT being significantly greater than baseline during stimulation with 50 or 100 Hz \(p\leq0.05\); mean current intensity 114.3±11.5 µA). (Figure 2)

Reinforcing effects of PAG stimulation

None of the 8 SNL rats acquired stable lever pressing for electrical stimulation of the PAG at any point during 10 days of training.
Figure 2. Effects of electrical stimulation of the periaqueductal grey (PAG) on paw withdrawal thresholds (PWT) in spinal nerve-ligated rats (SNL). The effects of electrical stimulation were determined following 60-sec of stimulation (40 stimulations per minute) in SNL rats (n=8) * Significantly different from baseline. # Significantly different from saline $P \leq 0.05$. 

Anti-Allodynic Effects of PAG Stimulation

Paw Withdrawal Threshold (g)

0  5  10  15

Frequency (Hz)

0  10  50  100

*
Discussion

In the current study the reinforcing effects of deep brain stimulation of the PAG was assessed in neuropathic rats. Ventral PAG stimulation significantly reversed mechanical allodynia following SNL in rats, yet produced no reinforcing effects assessed through acquisition of operant behavior using the ICSS paradigm. These findings suggest that alleviation of mechanical allodynia per se does not produce reinforcing effects in SNL rats.

It is possible that in addition to alleviating mechanical allodynia, stimulation of the ventral PAG produces aversive effects that interfered with acquisition of ICSS. During training sessions a portion of SNL rats appeared to produce avoidance-like behaviors during shaping which occurred immediately following stimulation. One possibility is that ventral PAG stimulation unintentionally stimulated aspects of the dorsal PAG, which when stimulated is aversive (Oliveras & Besson, 1988). This seems unlikely, however, since in some cases relatively low intensities (<150uA) elicited avoidance-like behavior.

It is also possible that long durations of ventral PAG stimulation are necessary to produce sufficient analgesia. If brief pulses, such as those used during ICSS, do not produce sufficient pain relief it may explain the failure to condition operant behavior with electrical stimulation of the PAG. For a similar reason most studies assessing drug self-administration utilize the intravenous route. During behavioral testing of the effects of PAG stimulation on mechanical allodynia, rats were stimulated for 60-sec prior to testing in order to allow them to habituate to stimulation. It is unclear if an anti-allodynic effect occurs immediately (<5
seconds) after stimulation begins; attempting to evaluate such a short-term effect is difficult when testing freely moving animals. Issues regarding duration of stimulation could be overcome by using the CPP paradigm, since noncontingent stimulation could be delivered over long time periods. From this standpoint, studies evaluating the reinforcing effects of DBS would benefit from the complementary use of operant and conditioning paradigms such as ICSS and CPP.

It is also possible that in the current study SNL rats did not suffer from spontaneous pain. Since it was hypothesized that relief of ongoing pain would produce reinforcing effects, if in fact spontaneous pain failed to develop it may explain why SNL rats failed to lever press for PAG ICSS. Nerve-injured rats do not display clear overt signs of ongoing pain, however operant and conditioning studies with intrathecal clonidine suggest the presence of ongoing pain in neuropathic rats. Spinal clonidine produces CPP (King et al., 2009) and maintains intrathecal self-administration selectively in nerve-injured rats (Martin, Kim, & Eisenach, 2006). Since spinal clonidine alleviates spontaneous pain in humans (Eisenach, DuPen, Dubois, Miguel, & Allin, 1995; Eisenach, Rauck, & Curry, 2003) it seems likely that its effects in rodents with nerve injuries reflects the presence and subsequent relief of spontaneous pain with clonidine. It is also possible that ventral stimulation only impacts elicited pain, such as mechanical allodynia, but does not influence spontaneous pain. Though possible, this seems unlikely given positive effects reported in patient populations (Gybel's & Kupers, 1990).
In conclusion, data from the current study reveal that electrical stimulation of the ventral PAG alleviates mechanical allodynia but did not produce reinforcing effects assessed with ICSS in SNL rats. This suggests that relief of allodynia per se does not produce reinforcing effects in SNL rats. It is also possible that the lack of reinforcing effects are due to other factors, including negative side effects associated with stimulation, time course of effect, or a lack of development or adequate relief of spontaneous pain following nerve injury with ventral PAG stimulation. These issues highlight the difficulties in addressing ongoing spontaneous pain with animal models of neuropathy, but also highlight the need for the complementary use of classical pain assessments (e.g., hypersensitivity) with those designed to address reinforcing aspects of pain relief.
Acknowledgements.

Supported by grants DA-022599 (TJM) and T32-DA-007246 (EEE) from the National Institute on Drug Abuse of the National Institutes of Health, Bethesda, Maryland.
References


Chapter VIII

Discussion
Summary

Chronic neuropathic pain is a major public health problem and treatment with prescription opioids, the fastest growing drug abuse problem in the United States (SAMHSA, 2008), is compromised over serious concerns regarding abuse potential (Compton & Volkow, 2006). Ascending pain pathways terminate supraspinally in cortical and limbic regions, which explains why pain comprises both sensory and affective components (Hunt & Mantyh, 2001). These termination sites indicate that pain and reward systems significantly overlap, yet little is known as to how these systems modulate activity of the other, and importantly how these interactions impact mechanisms of analgesia and/or addiction. To address this, the current work studied the effects of electrical stimulation of regions implicated in reward (VTA) and those implicated in analgesia (PVN, PAG) using a standard pain measure (mechanical allodynia) along with a measure of reward (ICSS).

Previous work suggests that neuropathic pain suppresses mesolimbic dopamine activity (Ozaki, Narita, Ozaki, Khotib, & Suzuki, 2004). It was therefore hypothesized that SNL rats would be less sensitive to the reinforcing effects of VTA ICSS, and that analgesics would attenuate these effects. Surprisingly, SNL rats responded for similar intensities and frequencies of VTA ICSS as control rats, and spinal analgesics failed to facilitate the reinforcing effects of VTA ICSS in SNL rats, suggesting that ongoing pain following nerve injury does not depress VTA dopamine neurons to a degree that can be measured using VTA ICSS. To determine if VTA ICSS is sensitive to pain manipulations, experiments were
undertaken using the paw incision model, which produces immediate and robust spontaneous pain early (<3 days) in the postoperative period (Brennan, Vandermeulen, & Gebhart, 1996). Paw incision surgery suppressed VTA ICSS in the early postoperative period (<3 days), and this occurred to a similar extent in both control and SNL rats. In contrast, hypersensitivity persisted for 10 days post surgery, similar to other reports (Zahn & Brennan, 1999), and was not different between groups. Collectively, these studies suggest that spontaneous pain, but not hypersensitivity, inhibits activity of mesolimbic dopamine circuitry. Furthermore, ongoing pain must be of sufficient magnitude to depress mesolimbic dopamine assessed with VTA ICSS, which occurs in the postoperative period but not following SNL in rats.

Previous research suggests that opioid stimulation of mesolimbic dopamine pathways is diminished following nerve injury in rodents (Ozaki, Narita, Iino, Miyoshi, & Suzuki, 2003; Ozaki et al., 2002). Since opioids facilitate VTA ICSS by stimulating mesolimbic dopamine (Wise, 1996) it was hypothesized that opioids would be less effective in facilitating VTA ICSS in SNL rats. In support of this, studies revealed that opioids (morphine, heroin, fentanyl, methadone, hydromorphone, and oxycodone) were less effective in facilitating VTA ICSS in SNL rats, yet cocaine’s effects were unchanged following nerve injury. This suggests that the reinforcing and abuse-related effects of opioids, which are mediated to some extent by stimulation of mesolimbic dopamine, are diminished during chronic pain states.
Based on previous work suggesting that relief of ongoing pain following nerve injury may be reinforcing in rats (Davoody et al., 2011; King et al., 2009; Martin, Kim, & Eisenach, 2006), it was hypothesized that electrical stimulation of brain regions implicated in analgesia would be reinforcing selectively in SNL rats. Electrical stimulation of the PVN and PAG alleviated mechanical allodynia in SNL rats, yet only PVN stimulation produced reinforcing effects measured with ICSS. However, non-injured rats also lever pressed for PVN ICSS, indicating that mechanisms other than analgesia mediate the reinforcing effects of PVN ICSS. Pharmacology studies suggest that central release of oxytocin may influence the reinforcing effects of PVN ICSS, which may be even more important following nerve injury since intrathecal atosiban diminished the reinforcing effects of PVN ICSS selectively in SNL rats. Morphine and cocaine facilitated PVN ICSS in normal and SNL rats, with morphine being more efficacious than cocaine, an effect that is distinct from MFB ICSS, and suggests that oxytocin release may interact with opioids, resulting in greater stimulation of limbic reward pathways.

**Conclusions & Future Directions**

Collectively, in the current studies the effect of nerve injury was limited in modulating ICSS. No differences were observed in neuropathic rats responding for ICSS (VTA, MFB, or PVN) compared to controls, and PAG stimulation failed to elicit reinforcing effects following SNL. It was expected that ongoing pain following SNL would lead to robust differences in responding for ICSS, and that these differences would further reveal the presence of ongoing pain in SNL rats.
The fact that this didn’t take place makes it entirely possible that ongoing spontaneous pain did not develop to a significant degree to modulate ICSS following nerve injury.

Neuropathic pain is debilitating in patients, leading to significant decreases in health related quality of life (Meyer-Rosberg et al., 2001). This implies that in order to simulate the clinical problem, preclinical models should similarly produce major decreases in quality of life in animal subjects. Though controversial, such an outcome of an animal model may be necessary for adequately assessing manipulations (pharmacological or other) aimed at producing clinically relevant effects against neuropathic pain. The current studies utilized the SNL model. Following SNL rats lost weight immediately in the postoperative period, but in days began to gain weight and by two weeks were back to their pre-operative weight and continued to gain weight normally thereafter. This type of recovery mirrors other surgical procedures performed (e.g., intrathecal catheter or intracranial electrode implantation) and indicates that these effects are due to acute tissue damage during the surgery and not nerve injury. In the weeks following surgery when behavioral testing is performed, SNL rats look surprisingly normal, and without prior knowledge even the most trained observer would be hard pressed to distinguish SNL from normal rats. Given this, it is not trivial to ask if SNL (along with other nerve injury models) produces relevant ongoing pain and/or significantly decreases quality of life in rodents, a topic that has been commented on elsewhere (Mogil, 2009).
Before concluding that better animal models are needed to produce greater severity of ongoing pain, it seems worthwhile to assess the effects of other currently used nerve injury models in modulating ICSS. A number of models have been developed, including partial sciatic nerve ligation (Seltzer, Dubner, & Shir, 1990) and chronic constriction injury (Bennett & Xie, 1988) among others, and it is possible that a different model may produce sufficient pain to modulate ICSS and other behavioral endpoints. It may also be worthwhile to look at chronic pain models other than nerve injury, including arthritic pain (Neugebauer, Han, Adwanikar, Fu, & Ji, 2007), cancer pain (Pacharinsak & Beitz, 2008) or spinal cord injury (Rozensweig & McDonald, 2004). Many of these models suffer from similar shortcomings to those described for nerve injury models, therefore these studies would be more than just proof of concept. Since humans have a different propensity for developing chronic pain based on the surgical procedure (Kehlet, Jensen, & Woolf, 2006), it seems likely that different chronic pain models would produce differing degrees of ongoing pain in animals.

Although it is difficult to measure ongoing spontaneous pain in animals, it is impossible to measure its absence following nerve injury. Negative results in detecting spontaneous pain can always be dismissed as a failure of the paradigm, and in this view it would appear that many approaches fail to measure ongoing neuropathic pain in animals. The typical approach taken in many studies is to measure a complex behavior in both nerve-injured and normal animals, and afterwards hypothesizing that differences between groups reflect ongoing pain. It may be more worthwhile to study how acute pain alters more complex behavioral
endpoints. This may seem like a strange suggestion given that acute models typically produce robust signs of spontaneous pain that are easily assessed. However, if chronic pain models are unable to produce such robust outward signs of ongoing pain, it may be worthwhile to determine how acute pain alters more complex and subtle behaviors, with the hope that these behavioral endpoints may similarly be affected by chronic pain models. One example of such an approach is the sophisticated use of the mouse grimace scale (Langford et al., 2010), which showed that acute pain produced distinct facial expressions in mice. It is concerning, however, that these authors failed to detect similar facial expressions in mice with neuropathic pain (Langford et al., 2010), which adds to the dilemma regarding severity of ongoing pain produced by nerve injury models.

It is overly optimistic to think that any single behavioral endpoint will serve as a magic bullet in answering questions related to measuring pain and analgesia related manipulations. In the case of analgesic development, it is important to know if a potential therapeutic produces desirable effects during pain states. This has led to increased interest in the use of novel approaches to study pain behavior, including paradigms that have been used for years in studying the rewarding aspects of drugs of abuse. These include drug self-administration, CPP, and in the current work ICSS. In this respect, these three behavioral measures provide important information regarding the reinforcing and motivational aspects pertaining to an environmental manipulation.

Drug self-administration and ICSS are much more direct measures of reward than CPP, since the animal actively engages in behavior to produce a stimulus.
Also, ICSS is typically performed at high rates and self-administration can be setup to induce high rates of responding (e.g., increasing the number of lever presses required to receive an infusion), which increases confidence that the behavior is mediated by the stimulus and is not performed randomly. In this respect, CPP is a much more indirect measure of reward, and since the behavior of interest (e.g., preference for a side previously paired with a manipulation) could be easily influenced by many factors (e.g., side bias, general variability) results often need to be viewed cautiously compared to operant behavior. In some cases, however, issues with learning can interfere with acquisition of operant behavior; such is the case when a drug has a slow onset of effect and/or long duration of action. Under these circumstances the CPP paradigm is useful because the animal can be placed in the side of the apparatus at a time in which the drug is expected to produce its desired effects, and therefore an association can still be made. It is possible that in this regard, electrical stimulation of analgesic regions of the brain may be better suited to produce CPP than operant behavior, if in fact the most desirable effects (e.g., analgesia) are acquired with long durations of stimulation, which appears to be the case with thalamic stimulation in rodents (Kupers & Gybels, 1993). In support of the notion that CPP may be more sensitive to reinforcing aspects of electrical stimulation than ICSS, a previous study reported that rats that did not lever press for MFB stimulation still developed conditioned place preference, and conversely those that did not lever press to turn off mesencephalic dorso-medial tegmentum stimulation (an
aversive stimulus) still developed conditioned place aversion (De Witte & Gewiss, 1987).

It is possible that modifications to the ICSS paradigm, compared to how it is typically used (e.g., lever pressing for stimulation) may make it more suited for assessing reinforcing effects of DBS following nerve injury. This is particularly true when assessing the reinforcing effects of stimulation of brain regions implicated in analgesia. An interesting approach would be to combine aspects of the CPP and ICSS paradigms. Using this approach rats would receive stimulation for their spatial positioning in a chamber instead of for lever pressing; in fact such a paradigm has been used with MFB stimulation (Ikeda, Moss, Fowler, & Niki, 2001). In the case of stimulation of reward pathways, the area that rats had to enter to receive stimulation typically made up a small percentage of the chamber and stimulation was triggered by activation of a photo beam (Ikeda et al., 2001).

One of the major benefits of using spatial location versus lever pressing is the increased likelihood of the animal producing the desired behavior. For instance, if stimulation is delivered contingent on residing in one half of the apparatus, rats would inevitably trigger stimulation during normal exploratory behavior, allowing them to sample the stimulus and increase the likelihood of associating stimulation with the target behavior. Furthermore, as learning developed the area producing stimulation could be reduced, requiring the animal to position itself in smaller areas; this would be ideal as it would increase experimenter confidence that the animal’s location within the stimulation zone is not coincidental.
Furthermore, this methodology would allow for detection of aversive effects associated with stimulation, which is more difficult to assess when lever pressing is used. For instance, animals may avoid regions associated with stimulation. In addition, to avoid concerns over the development of side biases, areas producing stimulation could be changed; rats should subsequently change their spatial position in order to continue or avoid stimulation depending on if the stimulation is reinforcing or aversive.

Collectively, this paradigm would use a similar behavioral endpoint (spatial location) as CPP, but rather than assessing a learned association, the behavior would be mediated by what is presently taking place, allowing for a more direct assessment of reward and/or aversion associated with stimulation. Therefore, this procedure encompasses two of the more beneficial aspects associated with CPP and ICSS, including ease of learning and direct assessment of reward, respectively. One limitation of this method is that its use is restricted to stimuli that can be turned on and off. Therefore, assessment of drug effects could not be assessed alone with this paradigm, but their modulation of stimulation effects could be assessed in a similar manner to traditional ICSS.

Thus far the case has been made that using different animal models along with refining the behavioral techniques employed may address some of the shortcomings reported in the current work. The goal of this would be to use models producing greater spontaneous pain along with more sensitive measures for its detection, which in turn would enhance our understanding of how nerve injury influences pain and reward circuitry. Even if such a pursuit is successful,
issues with interpretation still arise. Nerve injury produces behavioral effects other than hypersensitivity and spontaneous pain, including affective disorders such as anxiety and/or depression (Matsuzawa-Yanagida et al., 2008), and these cannot be dismissed when interpreting alterations in reinforcement mechanisms during chronic pain states.

One important question is whether stimulation of dopamine pathways produces effects beyond what is typically thought of as classic reward. Although the data indicate that stimulation of VTA or MFB failed to reverse hypersensitivity, it cannot be ruled out that stimulation produced desirable effects against other pain manifestations, such as reversal of a negative affective state. If this is the case, then limbic dopamine stimulation may induce affective analgesia (Franklin, 1989, 1998), an effect that would likely influence VTA ICSS to a greater extent in SNL rats.

The possible role of affective analgesia is even more pronounced when considering the reinforcing effects of PVN stimulation. Oxytocin has a well established function as an anxiolytic (Amico, Mantella, Vollmer, & Li, 2004) which may contribute to the reinforcing effects of PVN ICSS that are hypothesized to be related to centrally released oxytocin following PVN stimulation (Martinez-Lorenzana et al., 2008). If SNL rats in the current studies suffered from chronic anxiety, this may explain why intrathecal oxytocin had a greater impact in decreasing the reinforcing effects of PVN ICSS in SNL rats. In contrast, PAG stimulation, particularly stimulation of the dorsal PAG, is associated with increased anxiety, so much so that in some cases PAG stimulation has even
been used as a preclinical model of anxiety (Jung, Depoortere, & Oglesby, 2001). It is unclear if SNL rats didn’t lever press for PAG stimulation because it produced aversive effects (e.g., increase anxiety) or if it simply didn’t produce a desirable effect, but it would be expected that if nerve injury induces a negative affective state, then SNL rats may be especially sensitive to any undesirable effects associated with DBS.

Whether a manipulation alters ongoing pain or affective state during chronic pain states in animals may be indistinguishable. For instance, if ongoing pain drives negative affect, then manipulations which alleviate ongoing pain should consequently reverse negative affective state. Similarly, manipulations targeting elevations in mood and affect are likely to diminish the perceived aversive effects of ongoing pain, which may induce a state of indifference to the painful stimulus (Franklin, 1989). This is troublesome from a basic science standpoint addressing mechanisms underlying these behavioral measures; however from a translation standpoint it may not be important, as patients may benefit equally regardless of if the predominant effect is to shift affective state or block nociceptive input.

In conclusion, there is much to be optimistic about with respect to the use of operant and conditioning paradigms to study the effects of ongoing pain induced by preclinical models of chronic neuropathic pain. There is however need to make continued refinement of the animal models used (e.g., better selection of currently available or development of new models) as well as refinement of the dependent measures studied, which is needed to push the field forward in translating preclinical pain studies to the clinic. Ultimately such a goal would be to
discover targets which produce desirable effects under chronic pain states but are not reinforcing in the absence of pain in order to limit abuse. As the present work highlights, there are difficulties in distinguishing if the desired effects are limited strictly to pain relief (e.g., blocking nociceptive input), activation of reward pathways, a generalized shift in affective state, or some combination of each. Given the substantial overlap in these pathways it seems likely that the most beneficial therapies aimed at treating chronic pain will either directly or indirectly influence to some extent these endpoints, which may explain why opioids, despite their abuse concerns (Compton & Volkow, 2006) continue to be a mainstay in the treatment of chronic pain. It is hoped that information learned from the continued use and identification of behavioral measures implicated in ongoing pain will assist in the discovery of novel analgesic targets with diminished abuse liability and/or novel strategies to limit the abuse liability of opioids in treating chronic pain.
References


Curriculum Vita

Eric E. Ewan

- 2074 Walker Road  
- Winston-Salem, NC 27106  
- eewan@wfubmc.edu

Education

Aug., 2006  Graduate Student  Wake Forest University School of Medicine  
Physiology & Pharmacology

May, 2006  Bachelor of Arts  University of Wisconsin-Eau Claire  
Summa Cum Laude  Major: Psychology

Publications

- **Ewan, E.E. & Martin, T.J.** (accepted). Rewarding electrical brain stimulation in rats following peripheral nerve injury: Decreased facilitation by commonly abused prescription opioids. *Anesthesiology*


Presentations

**Symposia**

- “Toward an animal model of gambling.”
  * Assoc. for Behavior Analysis Annual Convention. Chicago, IL, Spring 2005.

**Poster Presentations**

- “Neuropathic pain alters the reinforcing effects of deep brain stimulation in rats.”

- “Neuropathic pain suppresses opioid activity within the mesolimbic dopaminergic system: Evidence from intracranial self-stimulation in nerve-injured rats.”
  * American Pain Society Annual Meeting. Baltimore, MD. Spring 2010

- “Neurochemical alterations in the amygdala during opioid self-administration in nerve-injured rats.”

- “Irreversible blockade of alpha-2 adrenergic receptors in the amygdala alters the behavioral effects of spinal clonidine in nerve-injured rats.”

- “Effects of DAMGO and DSLET in rats trained to discriminate 22 from 2 hours food deprivation.”
  * Student Research Day. Eau Claire, WI, Spring 2006

- “Preferences to work for fixed- vs. variable-reinforcer amount schedules.”
  * Student Research Day. Eau Claire, WI, Spring 2006
  * 14th National McNair Research Conference. Delavan, WI. Fall 2005.
  * Mid-American Assoc. for Behavior Analysis. Madison, WI, Fall 2005.
• “Economic demand for food and fat: Quantitative predictors of relative reinforcer efficacy.”
  * Mid-American Assoc. for Behavior Analysis. Madison, WI, Fall 2005.
  * Assoc. for Behavior Analysis Annual Meeting. Chicago, IL, Spring 2005.

• “Fixed- vs. variable-reinforcer amount schedules: Predictions of unit price.”
  * Mid-American Assoc. for Behavior Analysis. Indianapolis, IN, Fall 2004.

Student-Teaching Positions

• **Teaching Assistant.** Advanced Applied Behavior Analysis.
  *With Professor Kevin Klatt,
  *Psychology Department, University of Wisconsin-Eau Claire. Fall 2004.

• **Teaching Assistant.** Statistical Methods in Psychology.
  *With Professor Beverly Dretzke,
  *Psychology Department, University of Wisconsin-Eau Claire. Fall 2003.

Memberships & Distinctions

Membership

• American Pain Society. 2007 to present.
• Society for Neuroscience. 2007 to present.
• Ronald E. McNair Postbaccalaureate Achievement Program. 2004 to 2006.
• Phi Eta Sigma honor society. 2003 to 2006.
• Alpha Lambda Delta honor society. 2003 to 2006.

Distinctions

• Travel Award to American Pain Society Annual Meeting. 2010.
• UW-Eau Claire Dean's List. Fall 2001, Fall 2002 - Spring 2004
• Nominee of Psychology Department for George T. and Clayton T. Piercy Scholarship. 2004.