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Expression and Pharmacological Modulation of Pain-Depressed Behavior in Rats

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Expression and Pharmacological Modulation of Pain-Depressed Behavior in Rats

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

by

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List of Abbreviations

5-HT: Serotonin

CFA: Complete Freund's Adjuvant

COX: Cyclooxygenase

CPu: Caudate/Putamen,

DA: Dopamine

DAMP: Damage Associated Molecular Pattern

DOR: Delta Opioid Receptor

ICSS: Intracranial Self-Stimulation

IP: Intraperitoneal

KOR: Kappa Opioid Receptor

MOR: Mu Opioid Receptor

mRNA: Messenger Ribonucleic Acid

NE: Norepinephrine

norBNI: Norbinaltorphimine

NAc: Nucleus Accumbens

NAcC: Nucleus Accumbens Core

NAcSh: Nucleus Accumbens Shell

NNT: Number Needed to Treat

NSAID: Non-Steroidal Anti-Inflammatory Drug

PAMP: Pathogen Associated Molecular Pattern

PDYN: Prodynorphin

PFC: Prefrontal Cortex

qRT-PCR: Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

SC: Subcutaneous

THC: Δ9-tetrahydrocannabinol

VTA: Ventral Tegmental Area

List of Compounds

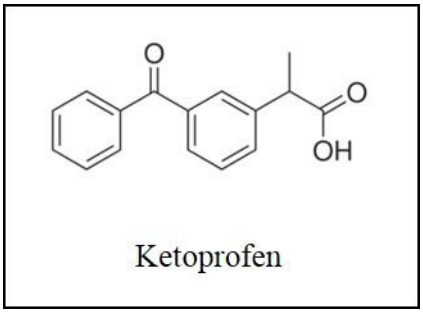
Noxious Stimuli:

- Lactic acid
- Complete Freund's Adjuvant (CFA)
- Formalin

Drugs:

- Ketoprofen
- Morphine
- U69593
- D-amphetamine
- Norbinaltorphimine (norBNI)
- Bupropion
- Delta-9-tetrahydrocannabinol (THC)
- Gabapentin

Structures of Compounds



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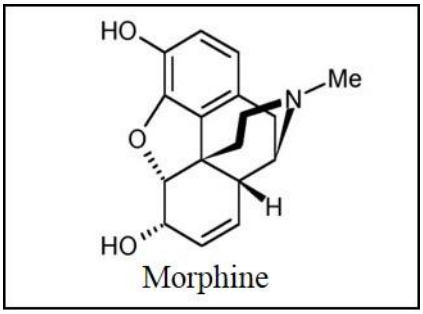

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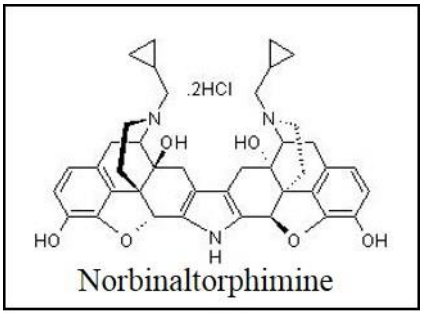

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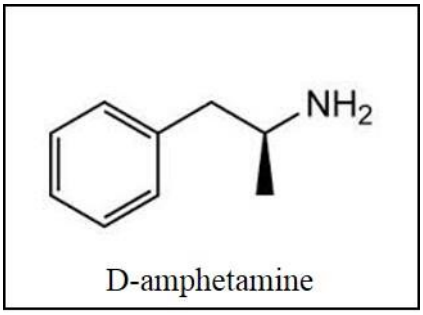

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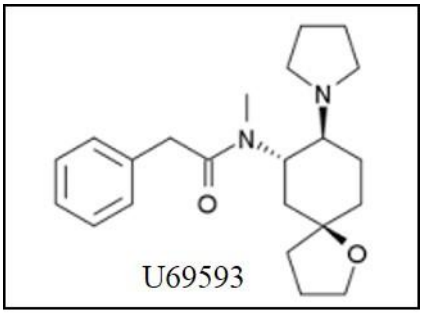

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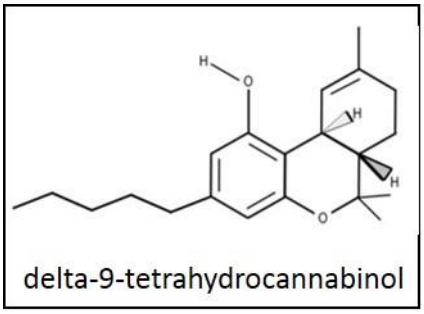
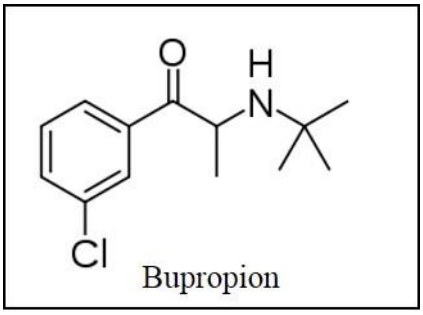
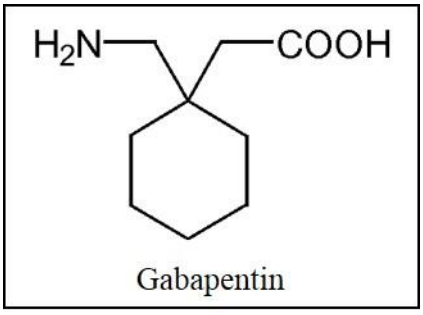


delta-9-tetrahydrocannabinol



Bupropion



Gabapentin

Abstract

EXPRESSION AND PHARMACOLOGICAL MODULATION OF PAIN-DEPRESSED BEHAVIOR IN RATS

By Michael D. Leitl

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

Virginia Commonwealth University, 2015

Advisor: S. Stevens Negus, Ph.D.

Pain is often associated with depression of behavior and mood, and relief of pain-related depression is a common goal of treatment. This goal of this dissertation was to conduct preclinical research experiments designed to address a set of three inter-related aims that examine the expression, mechanisms and treatment of pain-related depression of Intracranial Self-Stimulation (ICSS) in rats. First, studies evaluated the hypothesis that acute acid-induced depression of ICSS was mediated by a kappa opioid receptor mediated decrease in mesolimbic dopamine release in the nucleus accumbens. Results support a role for depressed mesolimbic dopamine release in pain-related depression of ICSS; however, a role for kappa opioid receptors is not supported. Second, studies evaluated the effectiveness of a more sustained inflammatory noxious stimulus (intraplantar CFA) and a sustained neuropathic stimulus (intraplantar formalin) to produce a long-term pain-related depression of ICSS, and the role of kappa opioid receptors in mediating this sustained pain-related depression of ICSS. Results indicated that only the neuropathic stimulus (formalin) was sufficient to produce sustained depression of ICSS, and as in the initial studies, our data did not support a role for kappa receptors in mediating this effect. Given the poor effectiveness of a kappa receptor antagonist to block acute or chronic pain-related

depression of ICSS, the final set of studies evaluated the pharmacology of representative drugs from five different classes of established or candidate analgesics (mu opioid agonists, non-steroidal anti-inflammatory drugs, monoamine uptake inhibitors, anticonvulsants, and cannabinoid agonists) to reverse the sustained depression of ICSS produced by formalin as a neuropathic stimulus. Results demonstrate the mu agonist morphine, the monoamine uptake inhibitor bupropion, the anticonvulsant gabapentin, and the cannabinoid agonist THC were able to reverse formalin-induced mechanical allodynia as a pain-stimulated behavior, but only the mu agonist morphine and the monoamine uptake inhibitor bupropion were effective to reverse formalin-induced depression of ICSS. These results provided additional evidence for dissociable drug effects in preclinical assays of pain-stimulated and pain-depressed behavior and also support further studies with monoamine uptake inhibitors with a dopaminergic component (like bupropion) for treatment of neuropathic pain.

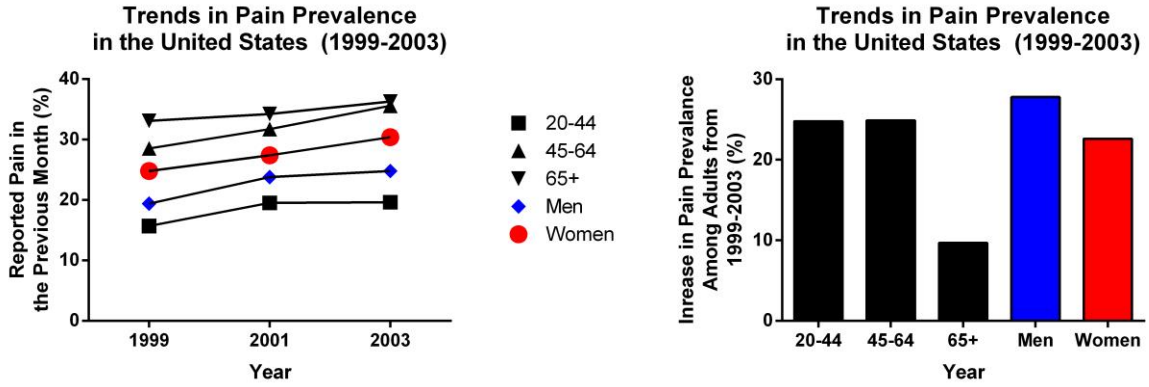
Chapter 1: Introduction

I. Definition, Clinical Expression, and Measurement of Pain in Humans

One of the vital functions of the nervous system is to provide information about the occurrence or threat of injury. The sensation of pain contributes to that function, and pain is described by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Accordingly, pain is appreciated to be a multi-dimensional experience that includes sensory, functional, and affective components. Pain affects approximately 100 million U.S. adults at a cost of $560-635 billion annually; this includes direct medical treatment costs and lost productivity (Institute of Medicine, 2011).

Figure I.1 shows trends of pain prevalence in the United States from 1999-2003 (adapted from Institute of Medicine, 2011). Data in the left panel show the percentage of adults reporting pain in the previous month (men and women) in respective age bins (20-44, 45-64, and 65+). Data suggest that age plays a role in the prevalence at which pain is reported; specifically, those 65 and older, and increasingly, those 45-64 years of age report pain at higher absolute percentages than those aged 20-44. Interestingly, while relative rates of younger people (20-44) reporting pain remain relatively low(er) compared to older patient populations, it is noteworthy that the increase in prevalence of people reporting pain in that age bin is increasing at rates consistent with older patient populations, as shown in the panel on the right. Additionally, there was an increase in the percentage of people reporting pain from 1999-2003, regardless of age or sex.

Figure I.1



Trends in pain prevalence in the United States from 1999-2003 (adapted from (Institute of Medicine, 2011).

The pervasive nature of pain and its burden on patients and society has stimulated efforts both to treat pain and to conduct research to understand its underlying neurobiological mechanisms. These efforts depend on strategies to measure the expression of pain and to quantify the effectiveness of clinical interventions. The most common dependent measures used by clinicians include metrics of the intensity of the pain a patient is experiencing (Institute of Medicine, 2011; Melzack, 1975; Cleeland & Ryan, 1994). In brief, pain intensity is generally measured on a unidimensional rating scale, such as a Visual Analog Scale (VAS) or an 11-point Numeric Rating Scale (NRS), in which patients are asked to verbally self-report their pain, subjectively, along a continuum from "no pain" (left end of a horizontal line in a VAS, 0 in a NRS) to "the worst imaginable pain" (right end of a horizontal line in a VAS, 10 in a NRS). VAS or numeric pain scores are considered valuable to clinicians as they provide a rapid determination of the pain intensity currently being experienced by a patient, and the primary goal of pain treatment is to reduce the pain intensity level on these scales.

In addition to pain intensity, effort has been placed on gathering more detailed information about the pain experience, including body regions that are most prone to (or most commonly reported to) produce pain. In a recent survey of U.S. patients reporting pain, seven distinct body regions stood out as being primary sources of pain, with low back pain being the most prevalent (or commonly reported) anatomical source of pain, as shown below in Table I.1, below.

Table I.1

Anatomical Location of Pain	**U.S. Average Adults I8 and Over (%)**
Low back pain	28.1
Knee pain	19.5
Severe headache or migraine	16.1
Neck pain	15.1
Shoulder pain	9.0
Finger pain	7.6
Hip pain	7.1

2009 Survey results of U.S. adults reporting pain in the preceding 3 month period: body regions affected (Institute of Medicine, 2011).

Although verbal and/or written measures and descriptions of pain intensity and identification of the anatomical regions where pain is reported to be produced are informative and can aid in treatment, additional attempts have been made to: 1) integrate these features into a

single questionnaire, and 2) further elucidate the quality and features of pain experiences. These attempts have taken several forms, and two pertinent examples of advancements include the Short Form of the McGill Pain Questionnaire (or SFMPQ; Melzack, 1987) and the Brief Pain Inventory (or BPI; Cleeland and Ryan, 1994).

In 1987, the Short Form of the McGill Pain Questionnaire (Melzack, 1987) was introduced. The SFMPQ, shown in Figure I.2, includes a *qualitative* assessment of pain by the patient, as well as a means for *quantification* by a clinician. The SFMPQ provides insight into 4 categories of pain that include: 1) intensity, 2) anatomical location, 3) adjectives or verbal descriptors that are said to be correlates of sensory qualities, and 4) adjectives or verbal descriptors that are said to be correlates of affective qualities. In the SFMPQ, pain intensity is indicated by a VAS in which the patient makes a mark along a (non-numeric) horizontal line that ranges from "no pain" (far left) to "worst possible pain" (far right). Pain location is indicated by marks on forward-facing and backward-facing diagrams of the human body. The patient is asked to draw on the diagram to identify where they are experiencing pain. Importantly, the SFMPQ expands upon these measures through inclusion of two additional components. Key additions to the SFMPQ include verbal descriptors (adjectives) that are considered to be correlates to sensory and affective features of pain quality. Specifically, 15 adjectives are provided, and (the first) 11 are considered correlates of sensory components of pain (including throbbing, stabbing, and splitting, among others) while (the last) 4 adjectives are considered correlates of affective components of pain quality (tiring-exhausting, sickening, fearful, and cruel-punishing). When filling out the SFMPQ, the patient indicates if they are experiencing each of the verbal descriptors or adjectives. The patient has the opportunity to leave each adjective blank (no response implies the adjective or feature of pain is not being experienced), or to rate each

adjective as mild, moderate, or severe. This form is a powerful tool for clinicians as guidance is provided to quantify the qualitative responses made by the patient. Specifically, with regard to pain intensity, the clinician assigns a number from 0-10 on the VAS based on the distance of the mark along the horizontal line. The clinician is further able to quantify the degree to which the pain being experienced by the patient is sensory or affective, and this is accomplished through assigning a numerical scoring system to responses to each of the verbal descriptors or adjectives. The SFMPQ includes guidance to the clinician that suggests summation of the ratings for the first 11 adjectives (0= no pain, 1= mild pain, 2= moderate pain, 3= severe pain; total possible equals 11*3=33) /33 (listed at the bottom of the form), and this summary score is considered to be a means of quantifying the sensory component of the pain being experienced. Similarly, summation of responses described above for the last 4 adjectives (4*3=12; score/12) provides the clinician an ability to quantify the affective nature of the pain. In summary, by using the SFMPQ, a clinician is able to determine 1) the intensity of pain being experienced, 2) where the pain is being experienced, and 3) if the pain is primarily sensory or affective in nature (or both). This additional information not only provides insight into the pain the patient is experiencing but also aids on how to treat the respective pain.

Figure I.2

Appendix IV (i)

SHORT FORM McGILL PAIN QUESTIONNAIRE and PAIN DIAGRAM

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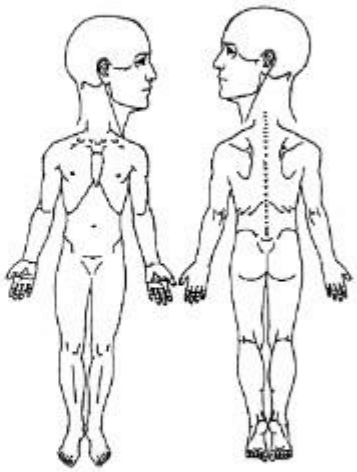
Date: _____

Name: _____

Check the column to indicate the level of your pain for each word, or leave blank if it does not apply to you.

		Mild	Moderate	Severe
1	Throbbing	_____	_____	_____
2	Shooting	_____	_____	_____
3	Stabbing	_____	_____	_____
4	Sharp	_____	_____	_____
5	Cramping	_____	_____	_____
6	Gnawing	_____	_____	_____
7	Hot-burning	_____	_____	_____
8	Aching	_____	_____	_____
9	Heavy	_____	_____	_____
10	Tender	_____	_____	_____
11	Splitting	_____	_____	_____
12	Tiring-Exhausting	_____	_____	_____
13	Sickening	_____	_____	_____
14	Fearful	_____	_____	_____
15	Cruel-Punishing	_____	_____	



Mark or comment on the above figure where you have your pain or problems

Indicate on this line how bad your pain is—at the left end of line means no pain at all, at right end means worst pain possible.

No Pain _____ Worst Possible Pain

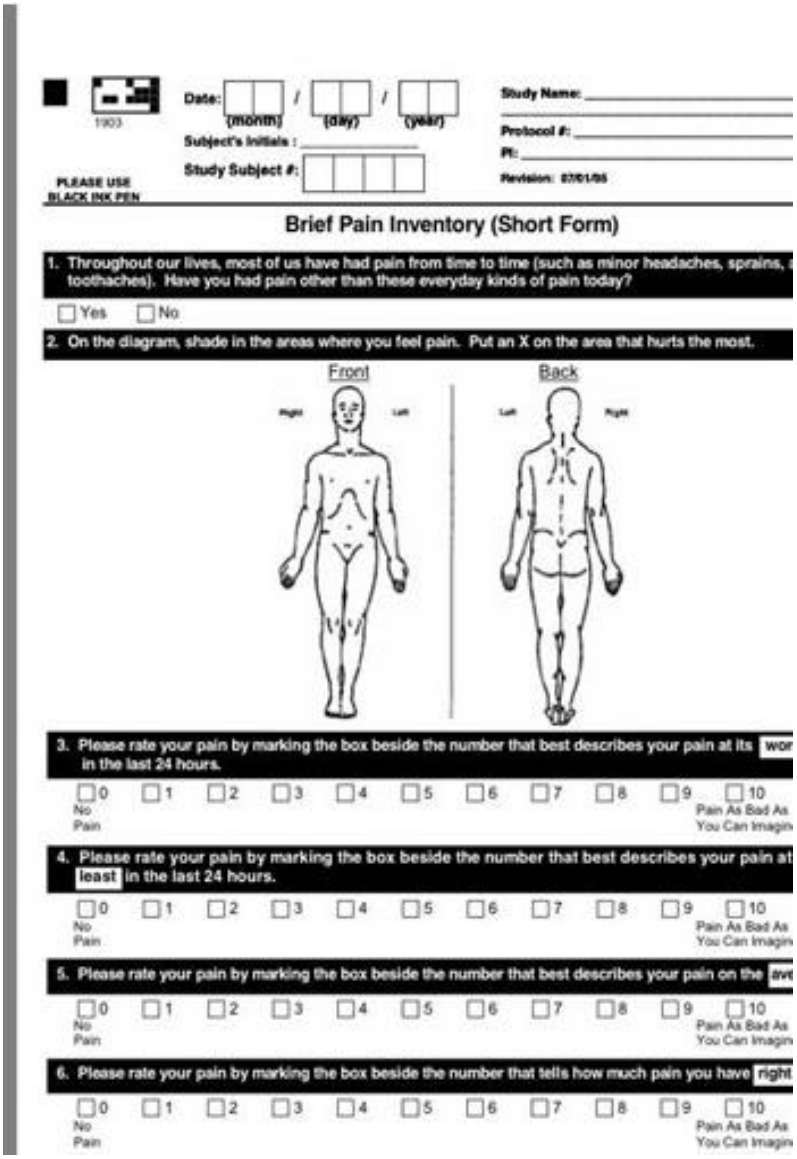
S /33 A /12 VAS /10

The Short Form of the McGill Pain Questionnaire (SFMPQ; Melzack, 1987)

A second clinical tool representing another major advancement for pain assessment was introduced in 1994, and is called the Brief Pain Inventory or BPI (Figure I.3; Cleeland and Ryan, 1994). The BPI seeks to characterize pain through additional and different means; specifically, the BPI specifically taps into the idea that clinically relevant pain states are also often accompanied by overt changes in behavior. The BPI advances upon the information gained in the SFMPQ through employment of a 0-10 scale to assess interference of pain on functional or quality-of-life dependent measures (including mood, walking ability, work, relationships with other people, sleep, and enjoyment of life), where "0" indicates no interference and "10" indicates complete interference. This information helps patients and clinicians further understand and recognize the implications of the pain being experienced on routine daily activities. This concept has been expanded upon, and it is now recognized that one of the most important categories of overt behavior associated with pain is functional impairment (Dworkin et al., 2005; Stewart et al., 2003).

Functional impairment and pain-related disabilities have profound impacts on individuals, and additionally result in productivity decreases in the economy and work-force. For example, more than half of 29,000 respondents to the American Productivity Audit telephone survey reported experiencing headache or musculoskeletal pain-related conditions during the previous 2 weeks (Stewart et al., 2003), and 1 in 8 respondents said their pain caused a loss of productive time. 1 in 14 said this lost work time exceeded 2 hours, and on average, respondents reported that their reduced performance amounted to 3.6 lost work hours per week (Stewart et al., 2003). Instruments such as the Brief Pain Inventory (Figure I.3, below) can be and are increasingly used to assess pain-related functional impairment (Cleeland and Ryan, 1994; Dworkin et al., 2005).

Figure I.3 (1/2)



Brief Pain Inventory (Short Form)

1. Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains, toothaches). Have you had pain other than these everyday kinds of pain today?

☐ Yes ☐ No

2. On the diagram, shade in the areas where you feel pain. Put an X on the area that hurts the most.

Front Back

3. Please rate your pain by marking the box beside the number that best describes your pain at its wor in the last 24 hours.

☐0 ☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐10
No Pain — Pain As Bad As You Can Imagin

4. Please rate your pain by marking the box beside the number that best describes your pain at least in the last 24 hours.

☐0 ☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐10
No Pain — Pain As Bad As You Can Imagin

5. Please rate your pain by marking the box beside the number that best describes your pain on the ave

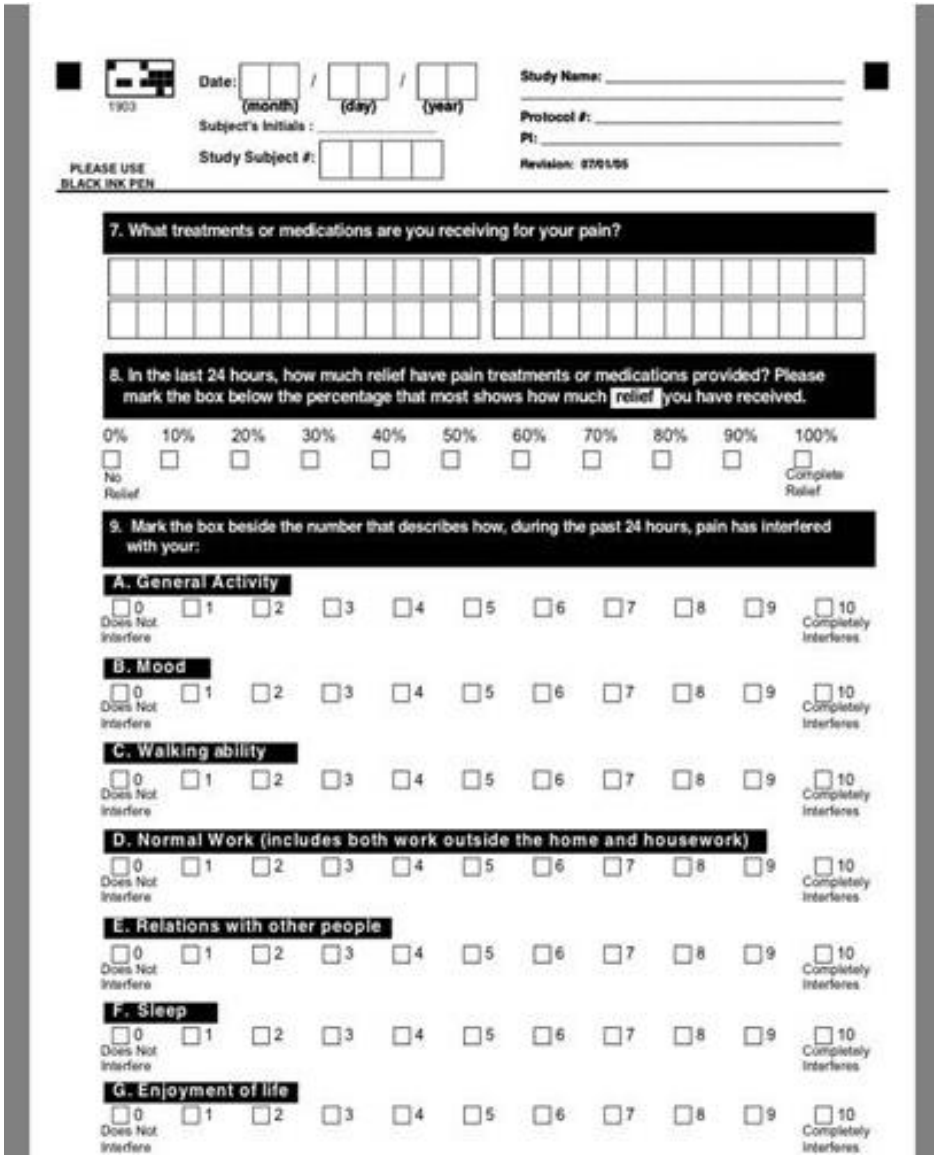
☐0 ☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐10
No Pain — Pain As Bad As You Can Imagin

6. Please rate your pain by marking the box beside the number that tells how much pain you have right

☐0 ☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐10
No Pain — Pain As Bad As You Can Imagin

The Brief Pain Inventory (BPI) is a clinical tool that incorporates functional aspects of pain and allows the patient an opportunity to provide additional information about how pain they are experiencing is affecting their daily routines and/or quality of life (Cleeland and Ryan, 1994).

Figure I.3 (2/2)



Date: (month) / (day) / (year)

Subject's Initials :

Study Subject #:

PLEASE USE BLACK INK PEN

Study Name:

Protocol #:

PI:

Revision: 07/01/05

7. What treatments or medications are you receiving for your pain?

8. In the last 24 hours, how much relief have pain treatments or medications provided? Please mark the box below the percentage that most shows how much relief you have received.

0% (No Relief) 10% 20% 30% 40% 50% 60% 70% 80% 90% 100% (Complete Relief)

9. Mark the box beside the number that describes how, during the past 24 hours, pain has interfered with your:

A. General Activity

B. Mood

C. Walking ability

D. Normal Work (includes both work outside the home and housework)

E. Relations with other people

F. Sleep

G. Enjoyment of life

0 (Does Not Interfere) 1 2 3 4 5 6 7 8 9 10 (Completely Interferes)

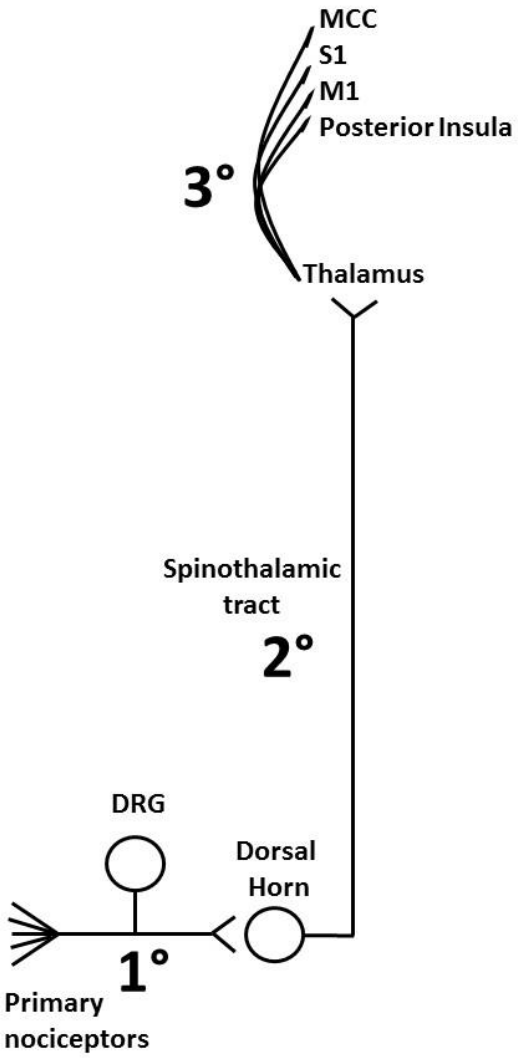
The Brief Pain Inventory (BPI) is a clinical tool that incorporates functional aspects of pain and allows the patient an opportunity to provide additional information about how pain they are experiencing is affecting their daily routines and/or quality of life

(Cleeland and Ryan, 1994).

II. Mechanisms of Pain

In order to provide clinically meaningful treatment of pain, it is useful to understand the different etiologies and neurobiological mechanisms that underlie the signaling and encoding of noxious stimuli and their role in the perception of pain. Advanced understanding of the neurobiology of different pain states has aided in not only the treatment of pain, but also in identifying patient populations that may (or may not) be likely to respond to particular pharmacotherapies. Three general categories of pain mechanisms have been identified: nociceptive pain, inflammatory pain, and neuropathic pain. It is reasonable to hypothesize that different etiologies of pain may be responsive to different pharmacotherapies, or alternative strategies, such as altering immune function.

Figure I.4



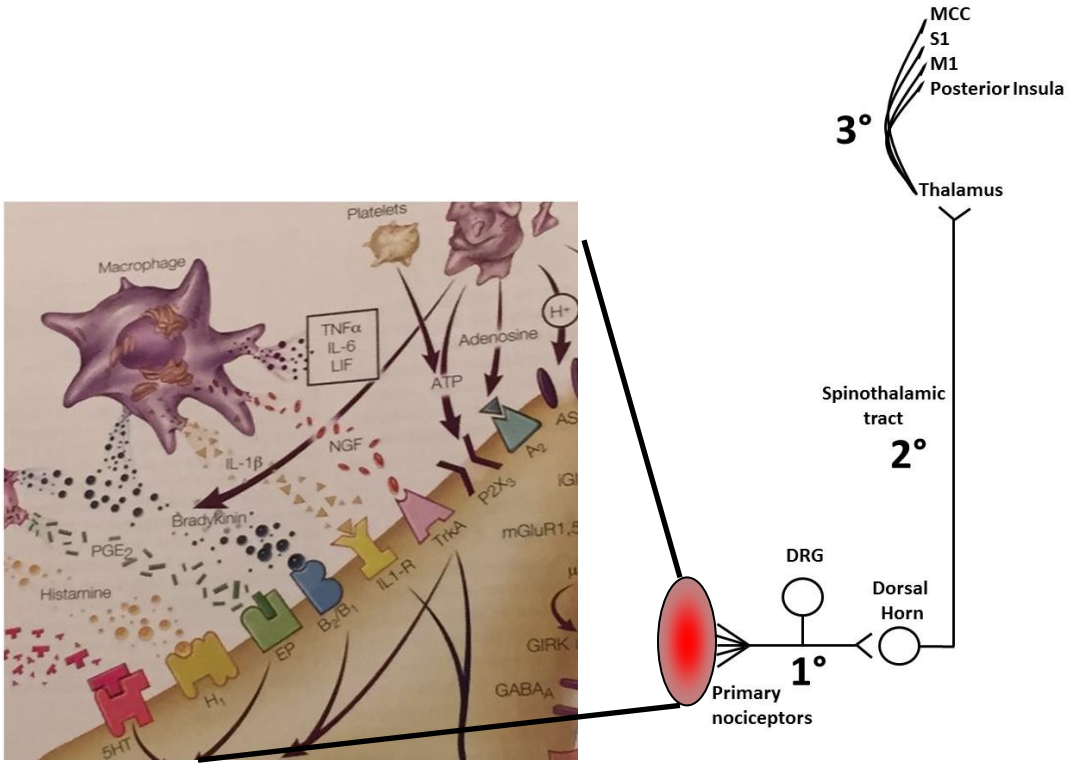
Nociception is the neural process of encoding noxious stimuli, which can be defined as stimuli capable of producing tissue damage. This diagram shows a simplified neural circuit of nociception composed of a chain of three neurons that project from the periphery to the brain. DRG=dorsal root ganglion, M1=primary motor cortex, MCC=medial cingulate cortex, S1=primary somatosensory cortex.

Nociception is the neural process of encoding noxious stimuli (which can be defined as stimuli capable of producing tissue damage), and Figure I.4 shows a simplified neural circuit of nociception. As a first step in nociception, a noxious stimulus activates primary (1°) nociceptors in the periphery. Primary nociceptors are pseudounipolar neurons with cell bodies that reside in dorsal root ganglia located along the spinal cord. One branch of these neurons projects peripherally to innervate skin, muscle or viscera, and the other branch projects centrally to the dorsal horn of the spinal cord. Primary nociceptors include both neurons with small, unmyelinated and slowly conducting axons (C fibers), as well as neurons with larger, myelinated and faster conducting axons (A∂ fibers). Under normal physiological conditions, some nociceptors respond exclusively to noxious stimuli, whereas other "wide dynamic range" neurons display graded responses from stimulus intensities the span the range from innocuous to noxious. Both types of neurons are considered to be "nociceptors" because they display different responses to innocuous vs. noxious stimuli. Stimulation of the peripheral terminals of primary nociceptors produces an action potential that travels centrally to promote release of the excitatory amino acid neurotransmitter glutamate, as well as of peptidic neurotransmitters (e.g. substance P), and subsequent activation of secondary (2°) nociceptors. Secondary nociceptors have cell bodies that reside in the dorsal horn of the spinal cord, and their axons project contralaterally and ascend via the spinothalamic tract to the ventrobasal nucleus of thalamus. Tertiary (3°) neurons have their cell bodies in thalamus, and they project to cortical targets including somatosensory cortex, anterior cingulate cortex, and insular cortex. In addition to this spino-thalamo-cortical neural circuit, other secondary nociceptors project from spinal dorsal horn to brainstem targets, and other tertiary neurons project from these brainstem targets to other higher centers including hypothalamus, amygdala and ventral tegmental area (VTA; Meyer et al., 2006; IASP, 2011).

Nociceptive pain is initiated by depolarization and subsequent activation of primary nociceptors by one of four modalities of noxious stimuli: thermal, chemical, mechanical and electrical. For example, hot and cold noxious stimuli activate primary nociceptors by activating TRPV1 and TRPM8 cation channels, respectively (Palkar et al., 2015; Okun et al., 2011). Chemical stimuli such as capsaicin can also activate TRPV1 channels, whereas other noxious chemical stimuli, such as acids, can activate members of the family of acid sensing ion channels (ASICS; Deval & Lingueglia, 2015; Karczewski et al., 2010; Deval E et al., 2013). Less is known about sensors to noxious mechanical stimulation, but a family of piezo proteins has recently been identified, and these proteins may contribute to mechanical nociception (Bagriantsev, et al., 2014). Lastly, electrical stimuli activate nociceptors and other primary afferents through direct depolarization of afferent terminals (Biurrun et al., 2011; Aasvang et al., 2015).

Nociceptive pain is localized to the site affected by the noxious stimulus, and it serves to detect, localize and limit tissue damage. The unpleasant experience of nociceptive pain is often associated with motor withdrawal reactions that remove the affected body part away from the noxious stimulus, and both nociceptive pain and withdrawal behaviors typically subside when the noxious stimulus is no longer in contact with the body. Examples of nociceptive pain include pain derived from a thermal source by touching a hot stove or from a chemical source after consumption of capsaicin, the pungent ingredient in chili peppers. An example of mechanical nociception and associated pain is the unpleasant sensation and associated withdrawal response that occurs after stepping on a sharp object.

Figure I.5

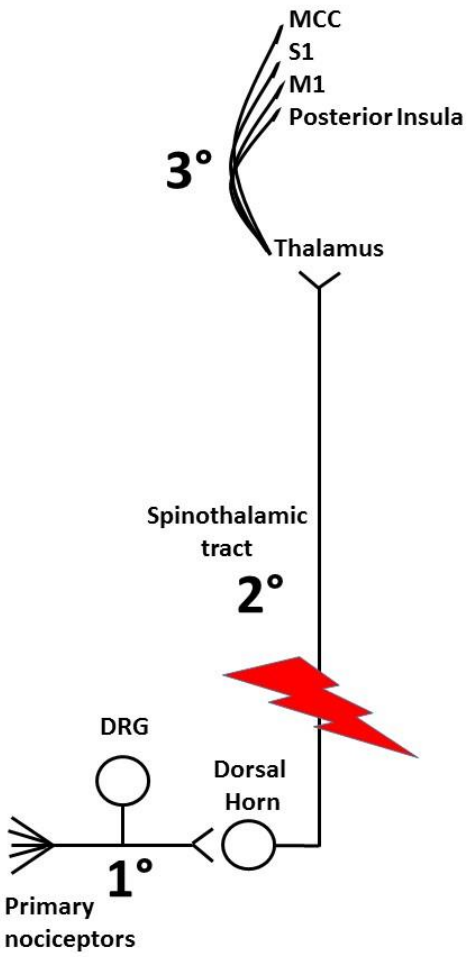


Inflammation is a process in response to an injurious stimulus and resulting presentation the immune system of damage-associated and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively). Inflammation is associated with four cardinal signs: 1) Rubor (redness) due to capillary dilation resulting in increased blood flow, 2) Tumor (swelling) due to passage of plasma from the blood stream to interstitial tissue, 3) Calor (heat) due to capillary dilation resulting in increased blood flow, and 4) Dolor (pain; mainly due to nociceptor sensitization and activation). Inflammatory pain is thought to result from sensitization of nociceptors by inflammatory mediators released at the site of inflammation.

Image on left adapted from (Meyer et al., 2007)

Inflammation is produced by infections or physical injuries that promote immune responses to products of host-cell damage (DAMPs) or to invading pathogens (PAMPs), or by auto-immune responses that target host proteins normally expressed in uninjured tissue. As one component of this immune response, inflammatory mediators such as cytokines, bradykinin, prostaglandins, and growth factors infiltrate the area of injury, bind to receptors expressed on sensory nerves, and sensitize nociceptors (Ren and Dubner, 2010; Meyer et al., 2006; Coruzzi et al., 2004). Behaviorally, this nociceptor sensitization can result in hypersensitive pain behaviors, and two terms are commonly used to describe this behavioral hypersensitivity. "Allodynia" indicates induction of pain behaviors by normally innocuous stimuli, and "hyperalgesia" indicates induction of exaggerated pain behaviors by normally noxious stimuli (IASP, 2011). Examples of inflammatory pain and associated behavioral hypersensitivity include sunburn produced by exposure to ultraviolet radiation and pain at the site of tissue incision after surgery. Inflammatory pain typically lasts longer than nociceptive pain because its time course is associated with the onset and offset of the inflammatory response at the site of injury rather than onset and offset of a particular external stimulus; however, it is usually localized to the site of injury and resolves once the injury heals. More sustained inflammatory pain can occur in cases of chronic inflammation, and in particular, in cases of auto-immune disorders such as rheumatoid arthritis.

Figure I.6



Neuropathy is defined as a disturbance of function or pathological change in a nerve, and neuropathy can result in abnormal activity in neural circuits that process noxious stimuli. For example, spinal cord injury can produce damage to spinothalamic tract neurons resulting in ectopic spontaneous discharge independent of normal nociceptor input. This abnormal activity in spinothalamic tract neurons would still activate higher order neurons in brain and may produce sensations of pain in the absence of a noxious stimulus.

Neuropathy results from an injury to a nerve and can result in neuropathic pain in some circumstances. Neuropathies can be considered mononeuropathies (one nerve damaged) or polyneuropathies (diffuse and bilateral nerve damage). Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system and can be either central or peripheral. Central neuropathic pain is caused by a lesion or disease of the central somatosensory nervous system while peripheral neuropathic pain is caused by a lesion or disease of the peripheral somatosensory nervous system (IASP, 2011).

Neuropathic pain may resemble inflammatory pain because spontaneous pain, allodnyia and/or hyperalgesia are often present. However, the underlying pathology is specifically in nerve tissue, as indicated above. After nerve injury, irregular regeneration injury can cause dysfunction in nociceptive processing and subsequent pain experiences by at least three different mechanisms. First, damaged primary, secondary or tertiary nociceptors may develop spontaneous patterns of ectopic activity that may produce sensations of spontaneous pain in the absence of an external stimulus. Second, nerve damage may promote plasticity in neuronal projections, such that reduced presynaptic inputs from the damaged nociceptor are replaced by inputs for other neurons not normally involved in nociception. As a result, innocuous stimuli that activate these new inputs may now activate higher order nociceptive neurons and produce allodynia. Lastly, reduced inputs from damaged nociceptors may decrease activation of local inhibitory interneurons and/or descending inhibitory neurons that normally inhibit nociceptive processing in neighboring receptive fields, resulting in disinhibited nociception and hyperalgesia. All neuropathic pain is chronic. A wide variety of pathological processes affecting peripheral nerves, sensory ganglia, spinal roots and CNS structures can induce neuropathic pain. These include trauma, vascular and metabolic disorders, bacterial and viral infection, inflammation,

autoimmune attack, genetic abnormalities and neurotoxins (Campbell and Meyer, 2006; IASP, 2011).

Symptoms of neuropathic pain can range from numbness, paresthesias and tingling to shooting, burning, sharp, electric shock-like pain sensations. Pain associated with spinal cord injury, peripheral diabetic neuropathy, chemotherapy-induced neuropathy, and cancer-related pain are all examples of neuropathic pain (Campbell and Meyer, 2006).

III. Current Treatments, Limitations, and Opportunities

Nociceptive pain is usually unintentional and unanticipated (e.g. stepping on a tack), and clinical intervention with prophylactic drug treatments is rare because the withdrawal responses that normally accompany nociceptive pain are highly adaptive. An exception is the use of local or general anesthetics to reduce nociceptive pain associated with medical procedures (e.g. tissue incision by a scalpel during surgery; drilling of a tooth as part of a dental procedure). Anesthetics are administered under close medical supervision, and as suggested by the word "anesthesia" (from Greek roots meaning "without feeling"), anesthetics decrease sensitivity not only to noxious stimuli but also to stimuli in other modalities. Anesthetics will not be considered further here.

By contrast, inflammatory and neuropathic pain are currently treated by a range of drugs that have varying degrees of effectiveness for producing "analgesia" (i.e. a selective decrease in sensitivity to pain; from Greek roots meaning "without pain"), and use of these drugs is often limited by side effects. The mechanisms, effectiveness, and principal side effects of the major drug classes that are used to treat pain and that were evaluated in this dissertation are described below.

The most widely prescribed drugs for treatment of moderate to severe pain are mu opioid receptor (MOR) agonists (Institute of Medicine, 2011). There are three major types of opioid receptors, the mu, delta and kappa opioid receptors (MOR, DOR, and KOR, respectively), and these receptors bind both endogenous neurotransmitters (e.g. ß-endorphin, met- and leu-enkephalin, dynorphin) and exogenous drugs such as morphine (Yaksh & Wallace, 2011; Finnerup et al., 2015). Opioid receptors are widely distributed in nociceptive circuitry as well as in other regions of the central and peripheral nervous system, the enteric nervous system, and the immune system (Yaksh and Wallace, 2011). All opioids used clinically as analgesics function primarily as agonists at MORs, and they are effective for treatment of many types of pain with particular effectiveness to treat severe types of inflammatory pain such as postsurgical pain (Yaksh and Wallace, 2011). Despite their clinical utility, the use of mu agonist analgesics is limited by side effects that include potentially lethal respiratory depression, nausea/emesis, constipation, and effects contributing to high abuse liability (Finnerup et al., 2015). Consistent with their clinical utility as analgesics, morphine and other mu opioid agonists block acid-induced depression of ICSS (Altarifi et al., 2014; Altarifi et al., 2012; Leitl et al., 2014)

NSAIDs represent an alternative class of effective analgesics. They are potent inhibitors of prostaglandin synthesis because they block cyclo-oxygenase (COX) enzymes necessary to produce prostaglandins. NSAIDs have four main pharmacological effects: anti-inflammatory, analgesic, antipyretic, and anti-thrombotic. Accordingly, NSAIDs are effective in treating acute postoperative inflammation and pain at the site of tissue incision (McQuay, 2007). There is also good evidence for the efficacy of oral NSAIDs in acute and chronic musculoskeletal pain (Derry et al., 2012; Haroutiunian et al., 2010). Most NSAIDs are appropriate for short-term use in inflammatory arthritic conditions such as rheumatoid arthritis and are reported to relieve pain of

headache, joint pain, and other mild-to-moderate pain syndromes (Ostor & Watson, 2013; McQuay, 2007). NSAIDs can also be used alone for mild-to-moderate pain or in combination with opioids for severe pain, and they are generally not habit forming. However, NSAIDs are effective only for pain states that involve an inflammatory component; they are not effective for treatment of either nociceptive pain or neuropathic pain (Finnerup et al., 2015). Long-term use of NSAIDs can cause a number of adverse effects including gastrointestinal bleeding, renal failure, and congestive heart failure (Singh, 1998; Coruzzi et al., 2004).

Given that there is a high co-morbidity of pain and depression, monoamine reuptake inhibitors represent a class of drugs rising in popularity for the treatment of pain. There are multiple subtypes of monoamine reuptake inhibitors with different selectivity for the serotonin (SERT), norepinephrine (NET), and dopamine (DAT) transporters. These subtypes include selective serotonin reuptake inhibitors (SSRIs; e.g. citalopram), serotonin (5-HT) norepinephrine (NE) reuptake inhibitors (SNRIs; e.g. duloxetine), NE and dopamine (DA) reuptake inhibitors (NDRIs; e.g. bupropion), triple reuptake inhibitors that block all three transporters (e.g. the experimental drug amitifadine), and mixed-action reuptake inhibitors including the subclass of tricyclic antidepressants (TCAs), named after their chemical structure. Despite the wide varieties of monoamine reuptake inhibitors, there is only evidence for limited effectiveness to treat neuropathic pain but not inflammatory pain or nociceptive pain (Atluri et al., 2015; Calandre et al., 2015; Dharmshaktu et al., 2012; Semenchuk et al., 2001). Additionally, monoamine uptake inhibitors have side effects including lethargy, loss of libido, and confusion (Gilron et al., 2015; Wang et al., 2015).

Gabapentin and anticonvulsants are commonly used to treat fibromyalgia and neuropathic pain, but generally have low effectiveness and high numbers needed to treat (NNT) (Guy et al.,

2014; Finnerup et al., 2015). Additionally, similar to monoamine uptake inhibitors, this class of drugs are also ineffective in the treatment of inflammatory pain or nociceptive pain (Chang et al., 2014). Furthermore, side effects limit the clinical utility of gabapentin and other anticonvulsant pharmacotherapies (Gilron et al., 2015; Kerstman et al., 2013; Phillips et al., 2010)

Δ9-tetrahydrocannabinol (THC) and other natural cannabinoids stem from the marijuana plant (*Cannabis sativa*), and many synthetic cannabinoids have also been developed. THC and other cannabinoids have been studied extensively with the intent of characterizing their therapeutic properties. Although the marijuana plant itself is widely used by humans, and although THC and other cannabinoids often appear analgesic in preclinical studies, there is poor evidence supporting its use in the clinic due to poor efficacy and high incidence of adverse effects (Finnerup et al., 2015; Lynch & Campbell, 2011; Ware et al., 2010).

IV. Preclinical Assessment of Pain and the Importance of Pre-Clinical Research in Drug Discovery and Development

Preclinical assays of pain have evolved over the years, but most studies probing the neurobiology of pain or response to analgesics have relied heavily, if not solely, on assays measuring "pain-stimulated behaviors." Pain-stimulated behaviors have been described as behaviors that increase in rate, frequency, or intensity in response to the delivery of a noxious or painful stimulus (Negus et al., 2006; Stevenson et al., 2006). Examples of pain-stimulated behaviors include (a) paw- or tail-withdrawal responses elicited by noxious thermal stimuli delivered by a light beam, hot plate, or hot-water bath, (b)stretching/writhing responses elicited by intraperitoneal (IP) injection of dilute acid, (c) paw flinching elicited by intraplantar administration of chemical irritants such as formalin, or (d) withdrawal responses elicited by

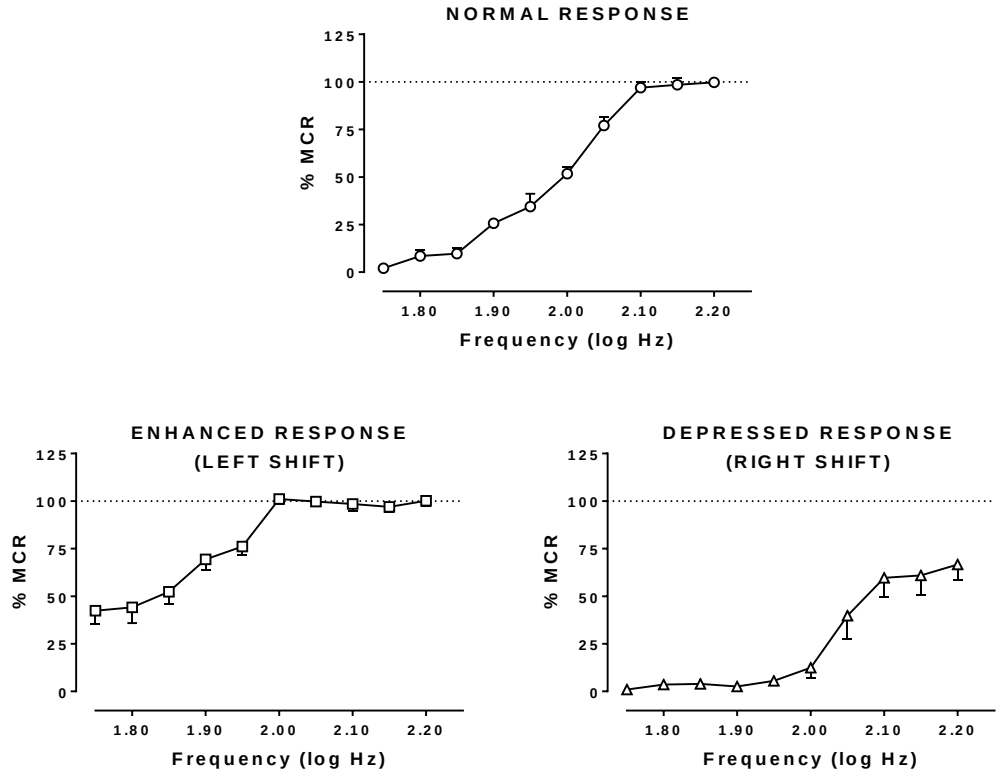
mechanical stimulation with von Frey filaments applied to tissue rendered hypersensitive by inflammation or neuropathy. In these assays, antinociception is implied if compounds decrease expression of the target behavior. Despite their wide use, these assays are associated with two significant and well-appreciated weaknesses: 1) drugs may produce a "false positive" decrease in pain-stimulated behaviors by producing motor impairment and a nonselective decrease in all behavior rather than a selective decrease in sensitivity to noxious stimuli, and 2) assays of pain-stimulated behavior do not model clinically relevant pro-depressant effects of pain (Negus et al., 2006; Pereira Do Carmo et al., 2009; Dworkin et al., 2009). Sedative drugs such as DA receptor antagonists, KOR agonists, and cannabinoid receptor agonists are prone to produce false-positive antinociception in assays of pain-stimulated behavior (Finn et al., 2004; Kwilasz and Negus, 2012). Moreover, pain-stimulated behaviors are rarely used clinically to diagnose pain or assess analgesic efficacy. Overall, excessive reliance on assays of pain-stimulated behaviors may have contributed to poor translation of results across species and clinical failures with candidate analgesics involving novel mechanisms in the recent past (Negus et al., 2006).

By contrast, assays of pain-depressed behavior measure ongoing behaviors that decrease in rate, frequency or intensity in the presence of a noxious stimulus. Feeding, locomotion and positively reinforced operant responding are examples of behaviors that can be depressed by pain, and preclinical assays of pain-related depression model pain-related depression of behavior and mood in humans. Antinociception is implied in these assays if compounds block or reverse pain-related depression of behavior and correspondingly increase expression of the target behavior (Negus et al., 2006; Stevenson et al., 2006). Assays of pain-depressed behavior have two attributes important to the assessment of candidate analgesics. First, antinociception is indicated by increases in the target behavior, and as a result, assays of pain-depressed behavior

are not vulnerable to false-positive effects caused by nonselective behavioral depression (Negus et al., 2010a; Negus et al., 2010b; Kwilasz and Negus, 2012). Second, assays of pain-depressed behavior may model pain-related functional impairment and/or depressed mood used to assess pain in both human and veterinary medicine (Cleeland and Ryan, 1994; Dworkin et al., 2005; Dworkin et al., 2009), and thus may provide insight into effects of candidate analgesics on these clinically relevant components of pain (Negus et al., 2010b). In view of these attributes, we have argued that assays of pain-depressed behavior may complement conventional assays of pain-stimulated behavior and increase the predictive validity of preclinical candidate analgesic assessment (Negus et al., 2006; Negus et al., 2010a).

One assay that has been used to generate baseline behavior for studies of pain-depressed behavior is intracranial self-stimulation (ICSS). ICSS is an operant behavioral procedure in which experimental subjects (usually rats) are equipped with an electrode that targets a component of the brain reward system, and subjects are trained to emit an operant response (e.g. pressing a lever) to receive pulses of brain stimulation delivered via the electrode (Negus and Miller, 2014). The magnitude of brain stimulation can be varied by manipulating stimulation frequency, and increasing frequencies of brain stimulation maintain a frequency-dependent increase in response rates. Thus, low brain stimulation frequencies maintain low rates of responding, whereas high rates of responding maintain high rates of responding. The graph that relates brain stimulation frequency (on the X-axis) to response rate (on the Y-axis) is called a frequency-rate curve, and a hypothetical example is shown in Figure I.7.

Figure 1.7



Hypothetical frequency-rate curves: (1) normal or baseline response, (2) hypothetical enhanced or left-shifted curve that might be produced by drugs of abuse (e.g. cocaine), and (3) hypothetical depressed or right-shifted curve that might be produced by a noxious stimulus.

Historically, the most common use of ICSS in preclinical behavioral pharmacology has been to evaluate neurobiology of brain reward systems and pharmacology of abused drugs. For example, most drugs of abuse (e.g. cocaine) increase low rates of ICSS maintained by low brain-stimulation frequencies and produce leftward shifts in ICSS frequency-rate curves (Negus and Miller, 2014; Figure I.7). Conversely, many aversive stimuli, including noxious stimuli that presumably produce aversive pain states, decrease high ICSS rates maintained by high brain-stimulation frequencies and produce rightward and/or downward shifts in ICSS frequency-rate curves (Negus, 2013; Figure I.7). Decreases in ICSS rates produced by noxious stimuli provide one example of pain-related depression of behavior, and drugs can be evaluated for their effectiveness to block or reverse pain-related depression of ICSS (Negus, 2013; Negus and Altarifi, 2013). For example, we have found in previous studies that ICSS can be depressed by IP injection of dilute lactic acid as an acute visceral noxious stimulus, and acid-induced depression of ICSS can be blocked by clinically effective analgesic drugs including both NSAIDs and mu opioid agonists. Conversely, acid-induced depression of ICSS is not blocked by drugs (e.g. centrally acting KOR agonists; DA receptor antagonists) that fail to produce clinically effective analgesia in humans but that do produce "false-positive" antinociception in conventional assays of pain-stimulated behavior. Studies in this dissertation used ICSS as the primary baseline behavior for studies of pain-depressed behavior. Major goals of the dissertation were to evaluate neural mechanisms of acute ICSS depression produced by IP acid and to evaluate sensitivity of ICSS to depression by other noxious stimuli thought to produce more sustained chronic inflammatory and neuropathic pain states.

Preclinical research remains essential to advance our understanding of how the body functions, and ultimately on how to treat disorders such as pain. Discovering and developing

drugs novel drugs that are safe and effective against the presence of hypersensitivities remains a worthwhile goal. While a fair amount of criticism has been attributed to poor "predictive validity" of animal models and pre-clinical research, it is important to be mindful of the fact that pain drug development can fail for numerous reasons including toxicity and dose-limiting side effects that are not always apparent in the model organism. Additionally, drugs fail in clinical trials because they fail to show improvement or otherwise differentiate from currently available treatment; there are a number of reasons this can occur, with one reason being a poor selection or patient population to study. Additional considerations that could be made include which animal models and endpoints the investigators feel is most relevant to the cohort of patients enrolling in the clinical study (Whiteside et al., 2013; Negus et al., 2006; Mogil, 2009).

V. Mechanisms of pain-related depression of ICSS

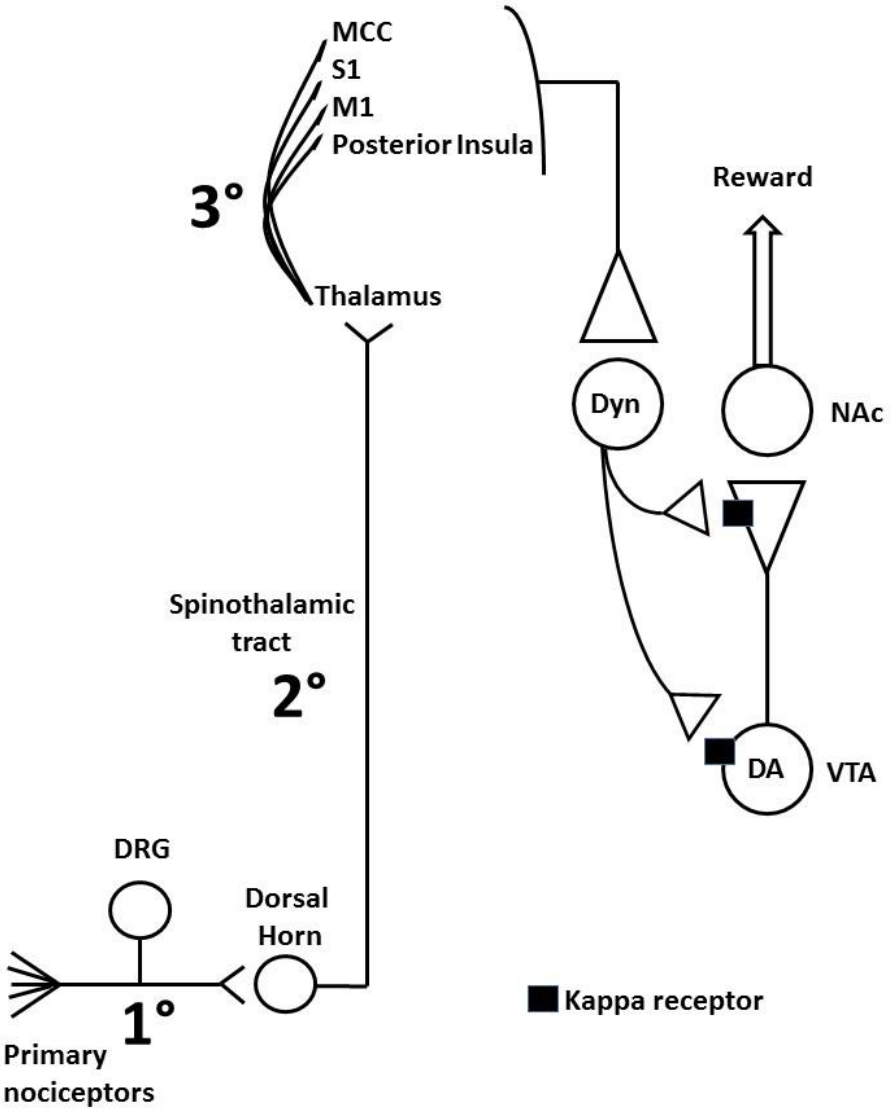
The mechanisms that underlie pain-related depression of behavior in general and of ICSS in rats in particular are not well understood. ICSS is mediated in part by activation of mesocorticolimbic DA neurons that originate in the VTA and project to terminal regions that include Nucleus Accumbens (NAc) (Stellar and Stellar, 1985; Wise, 2008). The mesolimbic DA system has a well-established role in mediating the rewarding effects of not only brain stimulation, but also natural reinforcers (e.g, preferred foods) and of abused drugs (e.g, stimulants and opioids) (Di Chiara and Imperato, 1988a; Wise, 2008). Depression of ICSS by noxious stimuli suggests that noxious stimuli may also depress signaling by mesolimbic DA neurons. This hypothesis is supported by other evidence that activity of DAergic neurons is negatively correlated with depressive dimensions of pain (Borsook et al., 2007; Jarcho et al., 2012; Wood PB, 2008). One goal of the present dissertation was to test the hypothesis that IP

acid-induced depression of ICSS would be associated with an analgesic-reversible depression of mesolimbic DA release as assessed by measures of extracellular NAc DA levels.

Pain-related depression of motivated behavior and of mesolimbic DA release could be mediated by a variety of different mechanisms, and elucidation of these mechanisms could suggest novel strategies for analgesic drug development. One possible mechanism is that noxious stimuli could activate endogenous dynorphin/kappa –opioid receptor systems as diagrammed in Figure I.8. In this schematic, cortical regions activated by noxious stimuli might provide excitatory input to a subset of NAc neurons that in turn project to and inhibit mesolimbic DA neurons in part by release of dynorphin. Dynorphin is an endogenous opioid peptide generated from the precursor preprodynorphin, and it functions as a moderately selective agonist for the kappa subtype of opioid receptors (Chavkin et al., 1982). VTA DA neurons express kappa– receptors on both their cell bodies and terminals, and activation of these kappa-receptors depresses neuronal activity and DA release (Knoll and Carlezon, 2010; Wee and Koob, 2010). For example, exogenous kappa -agonists such as salvinorin A and U69593 decrease both mesolimbic DA release and behaviors such as ICSS that depend on mesolimbic DA release (Carlezon et al., 2006; Di Chiara and Imperato, 1988a; Negus et al., 2012; Todtenkopf et al., 2004; Zhang et al., 2005). Moreover, recent studies suggest that some non-noxious stressors (e.g., forced swim in rats) activate dynorphin/kappa-systems to produce depressive-like effects that can be blocked by kappa-opioid receptor antagonists (Bruchas MR et al., 2010; Chartoff et al., 2009; McLaughlin et al., 2003). These findings have been interpreted to suggest that kappa-antagonists represent a novel class of candidate antidepressants that could block depressive-like effects associated with stress-induced dynorphin release, but it is unknown if induction of pain-states activate dynorphin/kappa-systems in a manner similar to non-pain stressors. Accordingly,

a secondary goal of this dissertation was to assess the degree to which pain-related depression of ICSS and NAc DA release might be (1) mediated by activation of endogenous dynorphin;/kappa-systems, and (2) blocked by a kappa-antagonist.

Figure I.8



Neural schematic of possible mechanisms whereby noxious stimuli could activate endogenous dynorphin/kappa–opioid receptor systems.

VI. Introduction to Data Chapters

This dissertation is composed of experiments designed to address a set of three inter-related aims that examine the expression, mechanisms and treatment of pain-related depression of ICSS in rats. Studies reported in Chapter II evaluated the hypothesis that acute acid-induced depression of ICSS was mediated by a KOR-mediated decrease in mesolimbic DA release in NAc. Our results support a role for depressed mesolimbic DA release in pain-related depression of ICSS; however, a role for KOR's is not supported. Studies reported in Chapter III evaluated the effectiveness of a more sustained inflammatory noxious stimulus (intraplantar CFA) and a sustained neuropathic stimulus (intraplantar formalin) to produce a long-term pain-related depression of ICSS, and the role of KORs in mediating this sustained pain-related depression of ICSS was also evaluated. We hypothesized that both inflammatory and neuropathic stimuli would produce long-lasting depression of ICSS, and that KORs would be more likely to play a role in sustained than acute pain-related depression of ICSS. Our results indicated that only the neuropathic stimulus (formalin) was sufficient to produce sustained depression of ICSS, and as in the initial studies, our data did not support a role for kappa receptors in mediating this effect. Given the poor effectiveness of a kappa receptor antagonist to block acute or chronic pain-related depression of ICSS, studies reported in Chapter IV evaluated the pharmacology of representative drugs from five different classes of established or candidate analgesics (mu opioid agonists, NSAIDS, monoamine uptake inhibitors, anticonvulsants, cannabinoid agonists) to reverse the sustained depression of ICSS produced by formalin as a neuropathic stimulus. We hypothesized that all drugs except the NSAID would produce dose-dependent and sustained reversal of formalin-induced ICSS depression. Although this hypothesis was supported for the reversal of formalin-induced mechanical allodynia as a pain-stimulated behavior, only the mu agonist

morphine and the monoamine uptake inhibitor bupropion were effective to reverse formalin-induced depression of ICSS. These results provided additional evidence for dissociable drug effects in preclinical assays of pain-stimulated and pain-depressed behavior and also support further studies with monoamine uptake inhibitors with a DAergic component (like bupropion) for treatment of neuropathic pain.

Chapter II: Acute expression of pain-depressed behavior, pharmacological modulation, and the role of endogenous kappa opioid system activation in rats.

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Introduction

Pain is often associated with depression of behavior and mood, and relief from pain-related depression is a common goal of treatment with analgesic drugs (Bair et al., 2003; Cleeland and Ryan, 1994; Dharmshaktu et al., 2012). The mechanisms of pain-related depression are not well understood. In preclinical studies, injury, disease, or treatment with experimental noxious stimuli can produce an analgesic-reversible depression of behaviors that include feeding (Kwilasz and Negus, 2012; Stevenson et al., 2006), locomotion (Cobos et al., 2012; Stevenson et al., 2009), burrowing (Andrews et al., 2012), and positively reinforced operant responding (Martin et al., 2004). For example, ICSS is an operant procedure in which subjects emit a learned response such as a lever press to earn pulses of electrical stimulation to brain reward areas (Carlezon and Chartoff, 2007b; Olds and Milner, 1954). Tissue acidosis is a cardinal component of inflammation that contributes to nociception and pain (Bray et al., 2013; Deval et al., 2013), and IP administration of exogenous acid functions as one type of physiologically relevant noxious visceral stimulus that depresses ICSS (Pereira Do Carmo et al., 2009; Negus, 2013). In addition, acid-induced depression of ICSS can be blocked by clinically effective analgesics

including both mu-opioid agonists such as morphine and nonsteroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen (Kwilasz and Negus, 2012; Negus et al., 2010a; Negus et al., 2010b; Pereira Do Carmo et al., 2009). Taken together, these observations suggest that acid-induced depression of ICSS may serve as a useful model for research on mechanisms of pain-related depression of behavior and brain reward systems.

ICSS is mediated in part by activation of mesocorticolimbic DA neurons that originate in the VTA and project to terminal regions that include NAc (Stellar and Stellar, 1985; Wise RA, 2008). The mesocorticolimbic DA system has a well-established role in mediating the rewarding effects of not only brain stimulation, but also natural reinforcers (eg, preferred foods) and of abused drugs (eg, stimulants and opioids) (Di Chiara and Imperato, 1988a; Wise, 2008). Depression of ICSS by noxious stimuli suggests that noxious stimuli may also depress signaling by mesolimbic DA neurons. This hypothesis is supported by other evidence that activity of DAergic neurons is negatively correlated with depressive dimensions of pain (Borsook et al., 2007; Jarcho et al., 2012; Wood, 2008). One goal of the present study was to test the hypothesis that IP acid-induced depression of ICSS would be associated with an analgesic-reversible depression of mesolimbic DA release as assessed by measures of extracellular NAc DA levels.

Pain-related depression of motivated behavior and of mesolimbic DA release could be mediated by a variety of different mechanisms, and elucidation of these mechanisms could suggest novel strategies for analgesic drug development. One possible mechanism is that noxious stimuli could activate endogenous dynorphin/kappa-opioid receptor systems. Dynorphin is an endogenous opioid peptide generated from the precursor preprodynorphin, and it functions as a moderately selective agonist for the kappa-subtype of opioid receptors (Chavkin et al., 1982).

VTA DA neurons receive inputs from dynorphinergic neurons and express κ-receptors, and activation of these kappa-receptors depresses neuronal activity and DA release (Knoll and Carlezon., 2010; Wee and Koob, 2010). For example, exogenous kappa-agonists such as salvinorin A and U69593 decrease both mesolimbic DA release and behaviors such as ICSS that depend on mesolimbic DA release (Carlezon et al., 2006; Di Chiara and Imperato, 1988b; Negus et al., 2012; Todtenkopf et al., 2004; Zhang et al., 2005). Moreover, recent studies suggest that some nonnoxious stressors (eg, forced swim in rats) activate dynorphin/kappa-systems to produce depressive-like effects that can be blocked by kappa-opioid receptor antagonists (Bruchas et al., 2010; Chartoff et al., 2009; McLaughlin et al., 2003). These findings have been interpreted to suggest that kappa-antagonists represent a novel class of candidate antidepressants that could block depressive-like effects associated with stress-induced dynorphin release. Accordingly, a secondary goal of the present study was to assess the degree to which acid-induced depression of ICSS and NAc DA release might be (1) mediated by activation of endogenous dynorphin/kappa-systems, and (2) blocked by a kappa-antagonist.

Materials and Methods

Subjects

Male Sprague–Dawley rats (Harlan, Frederick, MD) with initial weights of 285 to 400 g at the time of surgery were used for studies of ICSS and microdialysis. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 0600 h to 1800 h. Food and water were continuously available in the home cage. Animal maintenance and research were in compliance with National Research Council (2011) Guide for the Care and Use of Laboratory

Animals (National Academies Press, Washington, DC). In addition, animal-use protocols were approved by the Virginia Commonwealth University institutional animal care and use committee.

Noxious Stimuli and Drugs

Lactic acid (Spectrum Chemical, Gardena, CA), U69593 (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD), ketoprofen propionate (Spectrum Chemical), morphine sulfate (National Institute on Drug Abuse Drug Supply Program), d-amphetamine hemisulfate (Sigma Aldrich, St Louis, MO), and norBNI 2 HCl (synthesized by K Cheng and K Rice, National Institutes of Health, Bethesda, MD) were dissolved and/or diluted in sterile water. Lactic acid and U69593 were administered IP in a volume of 1 ml/kg. Ketoprofen, morphine, and d-amphetamine were administered subcutaneously in a volume of 1 ml/kg. Norbinaltorphimine (norBNI) was administered IP in a volume of 1.5 ml/kg. All doses were calculated using the salt forms of each drug as listed above.

Assay of Microdialysis

Surgery. A total of 30 rats were anesthetized with 2.5% isoflurane in oxygen until unresponsive to toe-pinch and secured in a sterotaxic apparatus (Kopf Instruments, Tujunga, CA). Guide cannulae (8 mm long, 0.5 mm outer diameter; CXG-8, Eicom, San Diego, CA) were implanted bilaterally and terminated 1 mm above the NAc (1.5 mm anterior to bregma, 1.8 mm lateral to midsaggital line, and 6.0 mm ventral to dura). A dummy cannula (CXD-8, Eicom) was inserted into each guide cannula to maintain patency. The guide cannulae were secured to the

skull with screws and dental acrylic. Animals were allowed to recover for at least 4 days before initiation of microdialysis testing.

Testing. On a test day, rats were briefly anesthetized with 2% isoflurane in oxygen, one dummy cannula was removed, and a microdialysis probe (CX-I-8-2, Eicom) with a 2-mm regenerated cellulose membrane (50 KDa molecular weight cutoff) was inserted through the guide cannula and into the NAc. The probe was connected to a two-channel liquid swivel (TCS2-23, Eicom), and the rat was placed in a clear plexiglass chamber (30 cm × 30 cm × 30 cm). Probes were perfused with a nonbuffered artificial cerebrospinal fluid solution (147 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$) at a rate of 1 μl/min. Following an equilibration period of at least 60 min, dialysate samples were collected into a 50 μl injector loop at 6-min intervals using an EAS-20s online autoinjector (Eicom) and immediately analyzed for DA concentrations by high-pressure liquid chromatography coupled to electrochemical detection (HTEC-500, Eicom). Preliminary experiments conducted by probe immersion into a known standard concentration of DA indicated a lag time of 24 min for dialysate to traverse the tubing from the probe to the electrochemical detector at the 1 μl/min flow rate (data not shown). The mobile phase consisted of 2% methanol (EMD, Gibbstown, NJ), 100 mM phosphate buffer (Sigma Chemicals, St Louis, MO), 500 mg/l 1-Decane sodium sulfonate (TCI America, Montgomeryville, PA), and 50 mg/l EDTA-2NA (Dojindo Laboratories, Kumamoto, Japan). DA was separated using a PP-ODS II reverse phase C18-column and detected using a graphite work electrode and an Ag vs AgCl reference electrode with an applied potential of +450 mV. DA was identified according to the retention time of the standard, and concentrations were quantified by comparison with peak heights of the standard concentration curve (0.01–100 pg per 10 μl) determined before each microdialysis experiment to ensure accuracy of standard retention times.

Resolution was sufficient to detect DA levels as low as 0.1 pg. DA levels were considered to have stabilized after collection of 10 consecutive baseline samples with <10% variability around the running mean. Testing was conducted using drugs, doses, and pretreatment times based on previous behavioral studies from our laboratory (see below; Altarifi and Negus, 2011; Negus et al., 2010a; Negus et al., 2012). Specifically, ketoprofen (3.2 mg/kg), morphine (3.2 mg/kg), or vehicle was administered subcutaneously 30 min before IP administration of dilute lactic acid (5.6% in 1 ml/kg), U69593 (0.56 mg/kg), or vehicle, and DA levels were recorded at 6-min intervals for 120 min. A higher ketoprofen dose (3.2 mg/kg) was used here than previously (1.0 mg/kg; (Negus et al., 2012) because of the higher intensity noxious stimulus (5.6% vs 1.8% lactic acid, respectively). The dose of U69593 was selected based on preliminary studies to identify a dose that produced depression of mesolimbic DA to a degree comparable to that produced by lactic acid. Following the experimental session, each rat (regardless of treatment) was administered 1.0 mg/kg d-amphetamine subcutaneously as a positive control to assess sensitivity of the preparation to a DA releaser (Baumann et al., 1994; Di Chiara and Imperato, 1988a). Rats were tested twice, once for each cannula, and treatments were counterbalanced. At the end of the experiments, rats were euthanized with CO_2, and the brains were removed and stored in 10% formalin. Brain tissue was blocked around the anatomic site of the guide cannula, and sections (100 μm thick) were made by vibratome through the area. The brain sections were then stained with cresyl violet. Anatomical probe placement was verified by gross visual and microscopic examination.

A 4-day experimental design was used in experiments with the κ-opioid antagonist norBNI to accommodate its slow onset and long duration of action (Bruchas et al., 2007; Endoh T et al., 1992), and two groups of rats were used to examine the effects of norBNI pretreatment

on the effects of IP acid and U69593, respectively. In each group, norBNI (32 mg/kg) was administered on day 1, and microdialysis test sessions were conducted on days 2 and 4. In one group of rats, the effects of vehicle and dilute acid (5.6% in 1 ml/kg), were examined on days 2 and 4, whereas in the second group, the effects of vehicle and U69593 (0.56 mg/kg) were examined on days 2 and 4. In each group, the order of testing was counterbalanced across rats. Microdialysis sampling sessions identical to those described above were conducted on test days. The effects of vehicle, lactic acid, or U69593 after norBNI pretreatment were compared with the effects of vehicle, lactic acid, or U69593 administered alone.

Histology. After microdialysis experiments, rats were euthanized by CO_2, and the brains were removed and placed in 10% formalin for at least 1 week. Tissue was blocked around the anatomic site of the guide cannula, and sections (100 μm thick) were made by vibratome through the area. The brain sections were then stained with cresyl violet. Anatomical placement was verified by gross visual and microscopic examination.

Data analysis. The primary dependent variable was the concentration of DA in each dialysate fraction. DA concentrations in each fraction for each rat were expressed as percent of the average of the 10 mean baseline concentrations before drug or vehicle administration. Two-way repeated measures ANOVA with treatment and time were used as the two main factors in Figure II.I (A). This experiment indicated that maximal decreases in DA levels were observed from 60 to 90 min after injection of acid, and a similar time course was observed for depression of mesolimbic DA by U69593. Accordingly, mean DA levels observed from 60 to 90 min after acid/U69593/vehicle injection were used for subsequent analyses. Treatment effects on mean DA levels during this time window were analyzed by one-way ANOVA. Significant ANOVAs were

followed by the Student–Newman–Keuls post hoc test, and significance was set a priori at the 95% level of confidence ($p<0.05$).

Assay of ICSS

Surgery. In all, 48 rats were anesthetized and secured in a sterotaxic apparatus as described above. The cathode of a stainless steel electrode (0.25 mm diameter, insulated except at flattened tip) was inserted into the medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to midsaggital line, and 8.8 mm ventral to skull). Three screws were placed in the skull, and the anode (0.125 mm diameter, uninsulated) was wrapped around one of the screws to act as a ground. The electrode was secured to the skull with dental acrylic. Animals were allowed to recover for at least 7 days before beginning ICSS training.

Apparatus. Experiments were conducted in sound-attenuating boxes that contained modular acrylic test chambers (29.2 × 30.5 × 24.1 cm^3) equipped with a response lever (4.5 cm wide, extended 2.0 cm through the center of one wall, and 3 cm off the floor), stimulus lights (three lights colored red, yellow, and green, positioned 7.6 cm directly above the response lever), a 2-W white house light, and an ICSS stimulator (MED Associates, St Albans, VT). Electrodes were connected to the stimulator via a swivel connector (model SL2C; Plastics One). The stimulator was controlled by computer software that also controlled all of the programming parameters and data collection (MED Associates).

Testing. After initial shaping of lever-press responding, rats were trained under a fixed-ratio 1 schedule of brain stimulation by using procedures similar to those described previously (Negus et al., 2010a; Negus et al., 2010b). During initial training sessions lasting 30 to 60 min, the white house light was illuminated, and responding produced electrical stimulation under a fixed-ratio 1 schedule of reinforcement. Under this schedule, each lever press resulted in the delivery of a 0.5-s train of square-wave cathodal pulses (0.1-ms pulse duration) and illumination for 0.5 s of the colored stimulus lights over the lever. Responses during the 0.5-s stimulation period did not earn additional stimulation. Initially, the frequency of stimulation was held constant at 126 Hz, and the stimulation intensity for each rat was adjusted gradually to the lowest value that would sustain a high rate of ICSS (≥30 stimulations/min). Frequency manipulations were then introduced, and the terminal schedule consisted of sequential 10-min components. During each component, a descending series of 10 current frequencies was presented, with a 60-s trial at each frequency. The frequency range extended from 158 to 56 Hz in 0.05-log increments. Each frequency trial began with a 10-s timeout, during which the house light was off and responding had no scheduled consequences. During the last 5 s of this timeout, five noncontingent stimulations were delivered once per second at the frequency available during that trial, and the lever lights were illuminated during each stimulation. This noncontingent stimulation was then followed by a 50-s 'response' period, during which the house light was illuminated, and responding produced electrical stimulation under the schedule described above. Training continued with presentation of three sequential components per day, and intensity was again adjusted as necessary until rats reliably responded for at least three and no more than six trials of all components for at least two consecutive days. Testing was conducted using drugs, doses, and pretreatment times identical to those used in microdialysis experiments, and as noted

above, these parameters were based on previous studies from our laboratory (Altarifi and Negus, 2011; Negus et al., 2010b). ICSS test sessions consisted of seven sequential components. The first component of each test session was considered to be an acclimation component, and data from this component were discarded. Data from the second and third 'baseline' components were used to calculate baseline parameters of frequency-rate curves for that session (see Data Analysis). After these baseline components, ketoprofen (3.2 mg/kg), morphine (3.2 mg/kg), or vehicle was administered subcutaneously 30 min before IP administration of dilute lactic acid (5.6% in 1 ml/kg), U69593 (0.56 mg/kg), or vehicle. Two sequential pairs of 10-min test components were then conducted 10–30 min and 70–90 min after the second injection. Testing was conducted twice per week (usually Tuesday and Friday), and the order of treatments for a group of rats was arranged according to a within-subject, counterbalanced design. Training sessions were conducted on other weekdays.

As in the microdialysis studies, a 4-day experimental design was used in experiments with norBNI, and two groups of rats were used to examine the effects of norBNI pretreatment on the effects of IP acid and U69593, respectively. In each group, norBNI (32 mg/kg) was administered on day 1, and ICSS test sessions were conducted on days 2 and 4 at 24 and 72 h after norBNI administration. In one group of rats, the effects of vehicle and dilute acid (5.6% in 1 ml/kg) were examined on days 2 and 4, whereas in the second group, the effects of vehicle and U69593 (0.56 mg/kg) were examined on days 2 and 4. In each group, the order of testing was counterbalanced across rats. ICSS test sessions identical to those described above were conducted on test days.

Data analysis. The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percentage of maximum control rate (%MCR), with the maximum control rate defined as the mean of the maximal rates observed during any frequency trial of the second and third baseline components for that session. Thus, %MCR values for each trial were calculated as (response rate during a frequency trial÷maximum control rate) × 100. For each test session, data from the second and third components were averaged to yield a baseline frequency-rate curve, and data from each pair of test components were averaged to yield test frequency-rate curves for the 10–30 min and 70–90 min time points. Baseline and test curves were then averaged across rats to yield mean baseline and test curves for each manipulation. For statistical analysis, results were compared by two-way, within-subject ANOVA, with treatment and ICSS frequency as the two factors. A significant ANOVA was followed by the Student–Newman–Keuls post hoc test, and the criterion for significance was set a priori at $p<0.05$.

As an additional summary measure of ICSS performance, the total number of stimulations per component obtained across all frequencies was determined, and the average number of stimulations per test component was expressed as a percentage of the average number of stimulations per baseline component during each session. These values were then averaged across rats in each experimental condition and compared by one-way ANOVA. A significant ANOVA was followed by the Student–Newman–Keuls *post hoc* test, and the criterion for significance was set *a priori* at $p<0.05$.

Quantitative real-time reverse transcriptase polymerase chain reaction (studies conducted in collaboration with S. Onvani and W.A. Carlezon Jr. at McLean Hospital, Harvard Medical School).

A total of 36 rats were used for qRT-PCR studies designed to assess the effects of acid noxious stimulus on endogenous prodynorphin (PDYN) and kappa-opioid receptor (KOR) mRNA. Rats were treated with IP saline vehicle or 5.6% lactic acid in a volume of 1 mg/kg and then killed by rapid decapitation after 1.5 h, 24 h, or 4 days (*N*=6 per treatment and time point). Brains were immediately extracted, rapidly frozen in −80 °C isopentane, and stored at −80 °C until analysis. Tissue punches were collected by sectioning frozen brains on a cryostat at −20 °C until the areas of interest were exposed. Bilateral punches of tissues from prefrontal cortex (PFC), NAc shell (NAcSh), NAc core (NAcC), caudate/putamen (CPu), and VTA were then collected and placed in Eppendorf tubes on dry ice. Total RNA was purified using PureLink RNA Mini Kit (Ambion). RNA quantity was measured (Nanodrop 2000, Thermo Scientific), and cDNA was synthesized from 500 ng of total RNA by using the iScript cDNA Synthesis Kit (Bio-Rad) and a ThermoHybrid iCycler (Thermo Scientific). The qPCR reactions were performed using the iQ SybrGreen Supermix (Bio-Rad) and the following primer pairs: KOR (5′-CTCCCAGTGCTTGCCTACTC-3′, and 5′-AGATGTTGGTTGCGGTCTTC-3′), PDYN (5′-ACTGCCTGTCCTTGTGTTCC-3′ and 5′-CCAAAGCAACCTCATTCTCC-3′), *β*-actin (NBA; 5′-AGGGAAATCGTGCGTGACAT-3′ and 5′-AAGGAAGGCTGGAAG AGAGC-3′), and Calnexin (CNX; 5′-GCTCTGGTCCATGACATCCG-3′ and 5′-CAGCATCTGCCCCACTACAC-3′). Primer specificity was confirmed by melt-curve analysis and polyacrylamide gel electrophoresis. The PCR reaction mix consisted of: 10 µl SybrGreen Supermix; 2 µl RNase/DNase-free water; 2 µl (3 µM) of each forward and reverse primers; and

4 µl (200 ng) cDNA template. Amplification was performed on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) under the following protocol: 95 °C for 5 min; 40 cycles at 94 °C for 15 s, 60 °C for 15 s, and 70 °C for 20 s. Read temperature for data collection was set between 81 °C and 86 °C for 15 s depending on the primer pair. A master cDNA mix was generated by mixing 10 µl of cDNA from all samples and used to produce a standard dilution curve on each plate. This was accomplished by serially diluting the master mix (1, 0.25, 0.0625, and 0.0156) and assigning to the undiluted sample an arbitrary concentration of 1 in the MyiQ Optical System Software (Bio-Rad). Two samples, each of which lacked either the cDNA template or reverse transcriptase enzyme, were run on every plate to control for reagent contamination and genomic DNA contamination, respectively. All samples were run in triplicate.

Data analysis. To normalize data, kappa-opioid receptor and PDYN values were divided by the average values of the two internal controls (β-actin and Calnexin). Values are reported as percent vehicle controls calculated as (normalized vehicle and experimental means/normalized vehicle group mean for the corresponding time point) × 100. Data for PDYN and KOR mRNA in each region were analyzed using two-way ANOVA with treatment and time as the two factors, followed by the Bonferroni post hoc test. The criterion for significance was set at $p<0.01$ to correct for multiple comparisons across the five different regions examined.

Results

Figure II.1 Shows the effects of IP lactic acid on NAc DA levels and ICSS. Over the course of the study, baseline extracellular DA levels in NAc were 1.0±0.1 pg per 5 µl. Figure II.1 (A) shows that IP vehicle injection had no effect on NAc DA, whereas IP injection of 5.6% lactic

acid produced a time-dependent decrease in DA. DA levels did not change for the first four samples (0–24 min) after acid injection because of the lag time for dialysate to travel from the probe to the electrochemical detector. Beginning at the time of treatment effect onset at the detector, DA levels decreased for the next seven samples (24–66 min after injection of vehicle or lactic acid, 0–42 min after treatment effect onset at the detector), and reached a nadir for the last five samples (60–90 min after injection, 36–66 min after treatment effect onset at the detector).

Treatment with the noxious lactic acid stimulus also depressed ICSS. Over the course of the study, the mean±SEM maximum control rate of ICSS was 59.8±1.5 stimulations/trial, and the mean total number of stimulations/component delivered across all frequencies was 291±13. Figure II.1 (B) shows that, relative to vehicle treatment, 5.6% lactic acid (IP) depressed ICSS from 10 to 30 min after acid injection, producing a rightward shift in the ICSS frequency-rate curve and significant decreases in ICSS at frequencies of 1.95–2.15 log Hz. ICSS was no longer significantly depressed 70–90 min after acid injection (data not shown). The relative time courses of these effects will be addressed in the Discussion section, but briefly, these data suggest that behavioral depression was associated with declining DA levels rather than with the absolute DA levels.

Figure II.2 Shows the effects of ketoprofen, morphine, and norBNI on lactic acid-induced depression of NAc DA levels and ICSS. Figure II.2 (A) shows that both the NSAID ketoprofen (3.2 mg/kg, SC) and the μ-opioid analgesic morphine (3.2 mg/kg, SC) blocked acid-induced depression of NAc DA at doses that did not significantly alter DA levels in the absence of the noxious stimulus. Figure II.2 (B) shows that the same doses of ketoprofen and morphine also

blocked acid-induced depression of ICSS at doses that did not significantly alter ICSS in the absence of the noxious stimulus.

Figure II.3 Shows that the kappa-agonist U69593 (0.56 mg/kg) decreased both NAc DA levels and ICSS (Figure II.III shows a more detailed display of these effects homologous to Figure II.I), and both effects of U69593 were completely antagonized by the kappa-antagonist norBNI. Ketoprofen failed to block U69593-induced depression of either NAc DA levels or ICSS. Morphine significantly attenuated U69593-induced depression of NAc DA levels, but did not alter U69593-induced depression of ICSS.

Figure II.4 Shows that a dose of norBNI that fully blocked U69593-induced depression of NAc DA and ICSS did not alter lactic acid-induced depression of either NAc DA or ICSS.

Figure II.5 Shows effects of IP lactic acid on transcript levels for PDYN and the kappa-receptor. Acid injection significantly increased PDYN expression in PFC at 4 days after acid injection. No significant changes in PDYN expression were observed in the PFC at earlier times, and no significant changes in PDYN expression were observed in NAcC, NAcSh, CPu, or VTA at any time. No significant changes in KOR expression levels were observed in any region at any time.

Figure II.6 Shows kappa receptor agonist-induced depression of NAc DA levels (A) and of ICSS (B) by U69593 (0.56 mg/kg, IP).

Figure II.7 Shows stability of basal DA levels prior to treatment for rats shown in Figure II.1 (A). A coronal section of the rat brain shows the positions of the microdialysis probes for all rats in the study (B). Numbers to the left of the sections indicate anterior-posterior position relative to bregma.

Summary

In agreement with previous studies, IP administration of dilute lactic acid served as a physiologically relevant noxious stimulus to produce an analgesic-reversible depression of ICSS, an operant procedure in which lever-press responding is maintained by electrical stimulation of a key brain reward area. The present study extended this finding in three ways. First, the acid noxious stimulus also depressed extracellular levels of the neurotransmitter DA in the NAc. The magnitude and valence of this effect was similar to the depression of DA release produced by some other dysphoric/aversive stimuli, such as kappa-agonists, and opposite to the stimulation of DA release produced by drugs of abuse and other rewarding/reinforcing stimuli. Accordingly, these data are consistent with the conclusion that depression of mesolimbic DA release may contribute to negative affective dimensions of pain. Second, acid-induced depression of NAc DA release was blocked by both NSAID and opioid analgesics. The sensitivity of acid effects to analgesic drugs provides further support for the potential relationship of these effects to affective dimensions of pain. Third, the acid noxious stimulus perturbed mesocorticolimbic PDYN expression, but the results of this study did not support a critical role for the dynorphin/kappa-opioid receptor system in mediating pain-related depression of ICSS and NAc DA. In particular, the kappa-antagonist norBNI failed to produce analgesic-like effects.

Figure Legends

Figure II.1 Pain-related depression of (A) NAc DA levels and (B) ICSS by IP injection of dilute lactic acid (5.6% in a volume of 1 ml/kg). (A) The abscissa shows time after injection of 5.6% lactic acid or its vehicle and the ordinate shows % baseline DA levels. The vertical line at 24 min indicates the lag time required for dialysate to traverse tubing between the dialysis probe and the electrochemical detector. The shaded area from 60 to 90 min shows asymptotic depression that was averaged for subsequent analyses. (B) The abscissa shows frequency of electrical brain stimulation (log Hz) and the ordinate shows ICSS rate expressed as percent maximum control rate (%MCR). Data were collected from 10 to 30 min after vehicle or acid injection, which corresponds to the time of declining DA levels in (A). In both panels, filled points indicate statistical significance of acid *vs* vehicle effects using two-way repeated measures ANOVA with Student–Newman–Keuls *post hoc* ($p<0.05$). Insets show average DA levels during the period indicated by the shaded region (a, expressed as % baseline DA levels) or average ICSS rates across all frequencies (B, expressed as % baseline stimulations across all frequencies). Asterisks (*) indicate a significant effect of acid as indicated by *t*-test ($p<0.05$). All data show mean±SEM from 5 to 7 rats.

Figure II.2 Effects of the analgesics ketoprofen and morphine on acid-induced depression of (A) NAc DA levels and (B) ICSS. (A) The abscissa shows treatment conditions and the ordinate shows % baseline DA levels from 60 to 90 min after administration. (B) The abscissa shows treatment conditions and the ordinate shows % baseline stimulations from 10 to 30 min after administration. Asterisks (*) indicate significantly different from vehicle conditions, and the symbol (#) indicates significantly different from acid alone as determined by one-way

ANOVA followed by the Student–Newman–Keuls *post hoc* test ($p<0.05$). All data show mean±SEM from 5 to 7 rats.

Figure II.3 Effects of the kappa-antagonist norBNI and of the analgesics ketoprofen and morphine on U69593-induced depression of (A) NAc DA levels and (B) ICSS. (A) The abscissa shows treatment conditions and the ordinate shows % baseline DA levels from 60 to 90 min after administration. (B) The abscissa shows treatment conditions and the ordinate shows % baseline stimulations from 10 to 30 min after administration. Asterisks (*) indicate significantly different from vehicle conditions, and the symbol (#) indicates significantly different from U69593 alone as determined by one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test ($p<0.05$). All data show mean±SEM from 5 to 7 rats.

Figure II.4 Effects of the kappa-antagonist norBNI on acid-induced depression of (A) NAc DA levels and (B) ICSS. (A) The abscissa shows treatment conditions and the ordinate shows % baseline DA levels from 60 to 90 min after administration. (B) The abscissa shows treatment conditions and the ordinate shows % baseline stimulations from 10 to 30 min after administration. Asterisks (*) indicate significantly different from vehicle conditions as determined by one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test ($p<0.05$). All data show mean±SEM from 5 to 7 rats.

Figure II.5 Pain-related modulation of PDYN and KOR mRNA expression levels in brain regions implicated in mood disorders as measured by qRT-PCR. Transcript levels of mRNA for (A-E) PDYN or (F-J) KOR in various components of midbrain DA systems. The abscissae show time after acid administration and the ordinates show transcript levels expressed as percent of vehicle control. Data were analyzed by two-way ANOVA followed by the

Bonferroni *post hoc* test. Asterisks indicate significant between-group differences within brain regions (**$p<0.01$). All bars show mean±SEM from 6 rats.

Figure II.6 Kappa receptor agonist-induced depression of NAc DA levels (A) and ICSS (B) by U69593 (0.56 mg/kg, IP). (A) Abscissa: Time after injection of U69593 or its vehicle. Ordinate: % Baseline DA levels. The vertical line at 24 min indicates the lag time required for dialysate to traverse tubing between the dialysis probe and the electrochemical detector. The shaded area from 60-90 minutes shows asymptotic depression that was averaged for subsequent analyses. (B) Abscissa: Frequency of electrical brain stimulation (log Hz). Ordinate: ICSS rate expressed as percent maximum control rate (%MCR). Data were collected from 10-30 min after vehicle or U69593 injection, which corresponds to the time of declining DA levels in Panel A. In both panels, filled points indicate statistical significance of U69593 vs. vehicle effects using two-way repeated measures ANOVA with Student-Newman-Keuls post-hoc test ($p<0.05$). Insets show average DA levels during the period indicated by the shaded region (A, expressed as % baseline DA levels ± SEM) or average ICSS rates across all frequencies (B, expressed as % baseline stimulations across all frequencies ± SEM). Asterisks indicate a significant effect of U69593 as indicated by t-test ($t<0.05$). All data show mean±SEM from 5-7 rats per treatment.

Figure II.7 Stability of basal DA levels prior to treatment and positions of the microdialysis probes for all rats. (A) Abscissa: Sample number (6 min consecutive intervals). Ordinate: % Baseline DA levels prior to starting any treatment manipulations. Error bars show SEM. (B) Coronal section depictions of the rat brain showing the positions of the microdialysis probes. Numbers to the left of the sections indicate anterior-posterior position relative to bregma.

Figure II.1

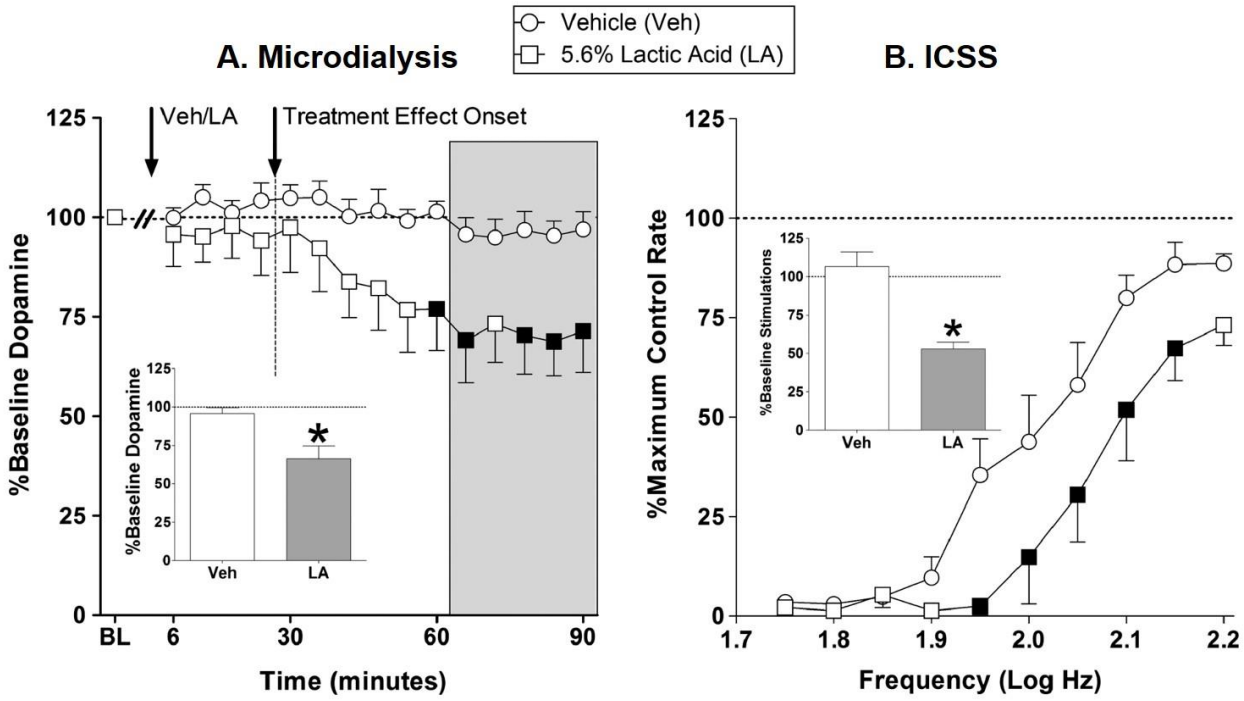
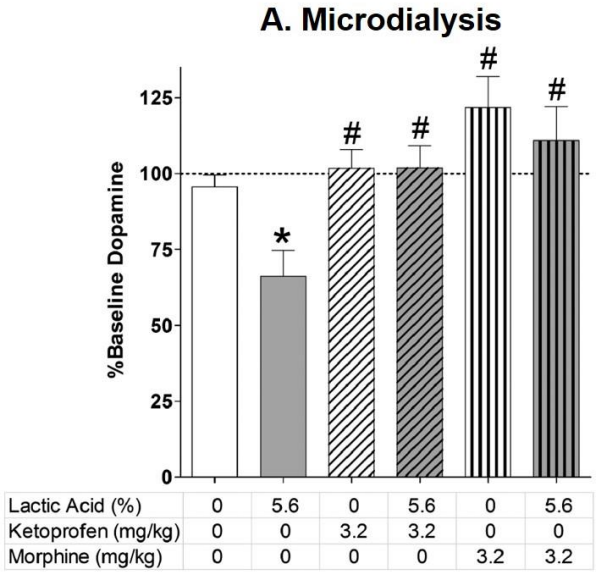

Vehicle (Veh)
5.6% Lactic Acid (LA)
A. Microdialysis
B. ICSS
Veh/LA
Treatment Effect Onset
%Baseline Dopamine
Time (minutes)
BL
6
30
60
90
Veh
LA
%Maximum Control Rate
Frequency (Log Hz)
%Baseline Stimulations

Figure II.2


A. Microdialysis
%Baseline Dopamine
125
100
75
50
25
0
*
#
#
#
#
Lactic Acid (%) 0 5.6 0 5.6 0 5.6
Ketoprofen (mg/kg) 0 0 3.2 3.2 0 0
Morphine (mg/kg) 0 0 0 0 3.2 3.2

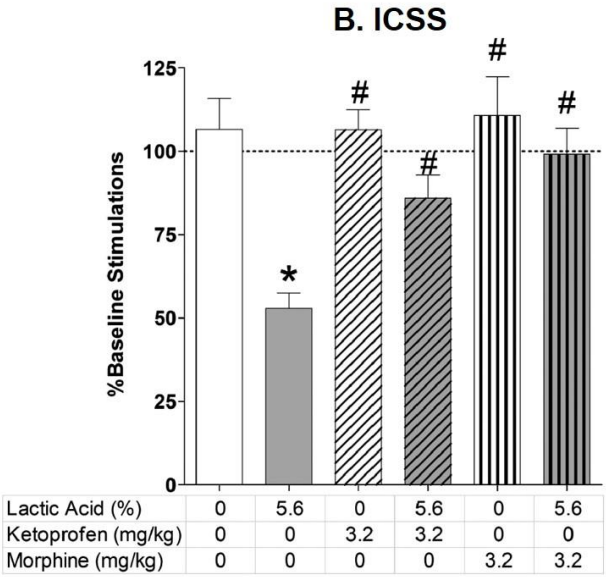
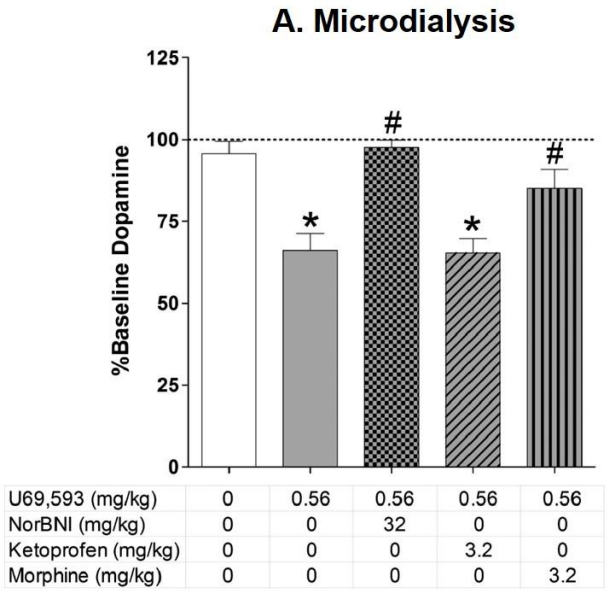
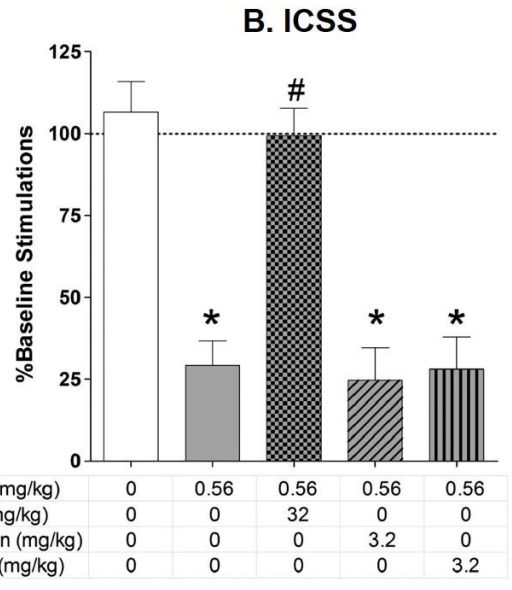

B. ICSS
%Baseline Stimulations
125
100
75
50
25
0
*
#
#
#
#
Lactic Acid (%) 0 5.6 0 5.6 0 5.6
Ketoprofen (mg/kg) 0 0 3.2 3.2 0 0
Morphine (mg/kg) 0 0 0 0 3.2 3.2

Figure II.3

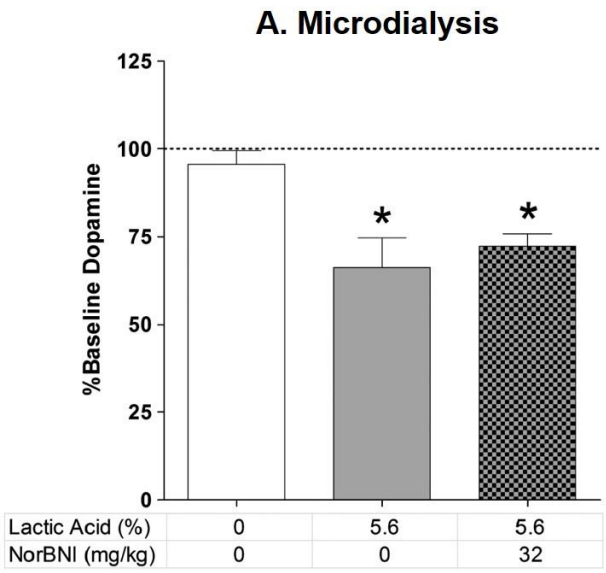


U69,593 (mg/kg)	0	0.56	0.56	0.56	0.56
NorBNI (mg/kg)	0	0	32	0	0
Ketoprofen (mg/kg)	0	0	0	3.2	0
Morphine (mg/kg)	0	0	0	0	3.2



U69,593 (mg/kg)	0	0.56	0.56	0.56	0.56
NorBNI (mg/kg)	0	0	32	0	0
Ketoprofen (mg/kg)	0	0	0	3.2	0
Morphine (mg/kg)	0	0	0	0	3.2

Figure II.4


A. Microdialysis
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125
100
75
50
25
0
*
*
Lactic Acid (%) 0 5.6 5.6
NorBNI (mg/kg) 0 0 32

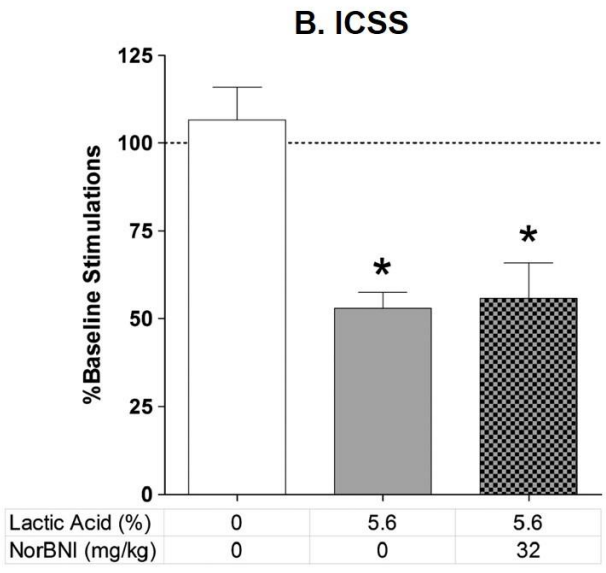

B. ICSS
%Baseline Stimulations
125
100
75
50
25
0
*
*
Lactic Acid (%) 0 5.6 5.6
NorBNI (mg/kg) 0 0 32

Figure II.5

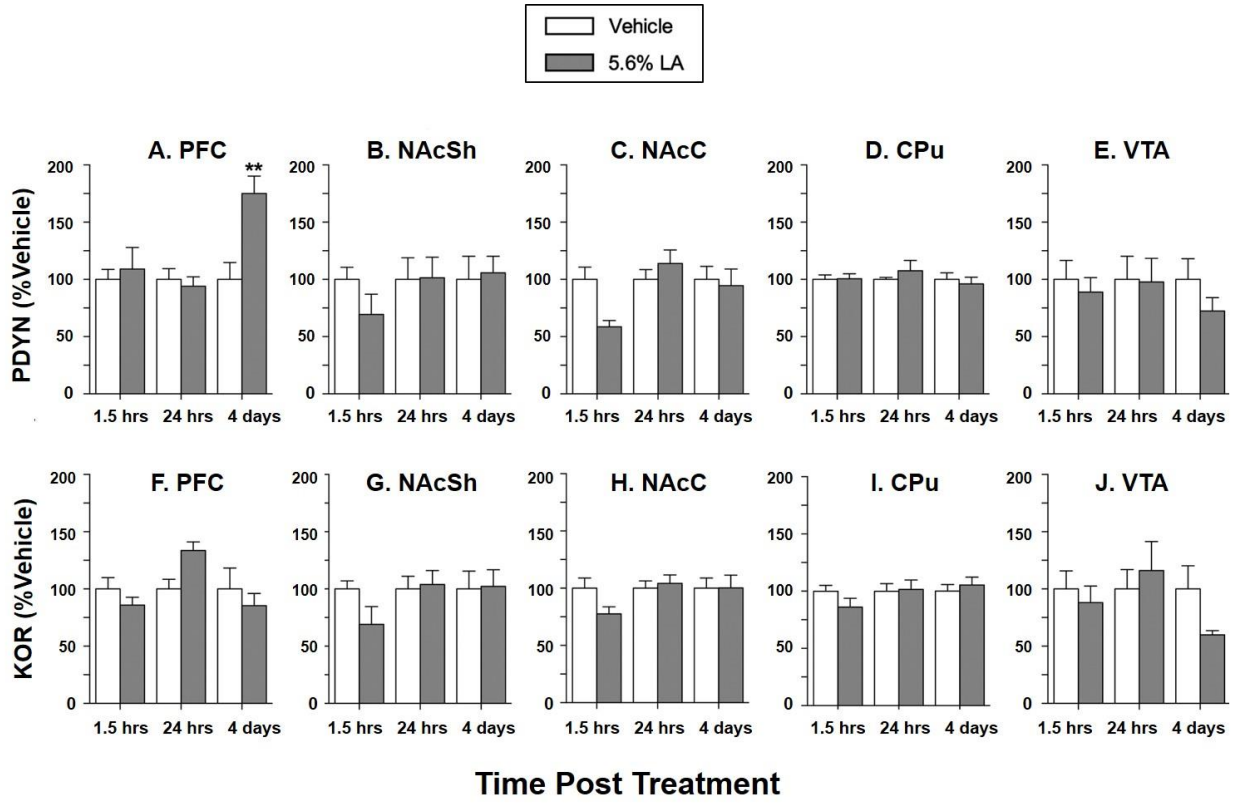

Vehicle
5.6% LA
A. PFC
B. NAcSh
C. NAcC
D. CPu
E. VTA
F. PFC
G. NAcSh
H. NAcC
I. CPu
J. VTA
**
PDYN (%Vehicle)
KOR (%Vehicle)
0
50
100
150
200
1.5 hrs
24 hrs
4 days
Time Post Treatment

Figure II.6

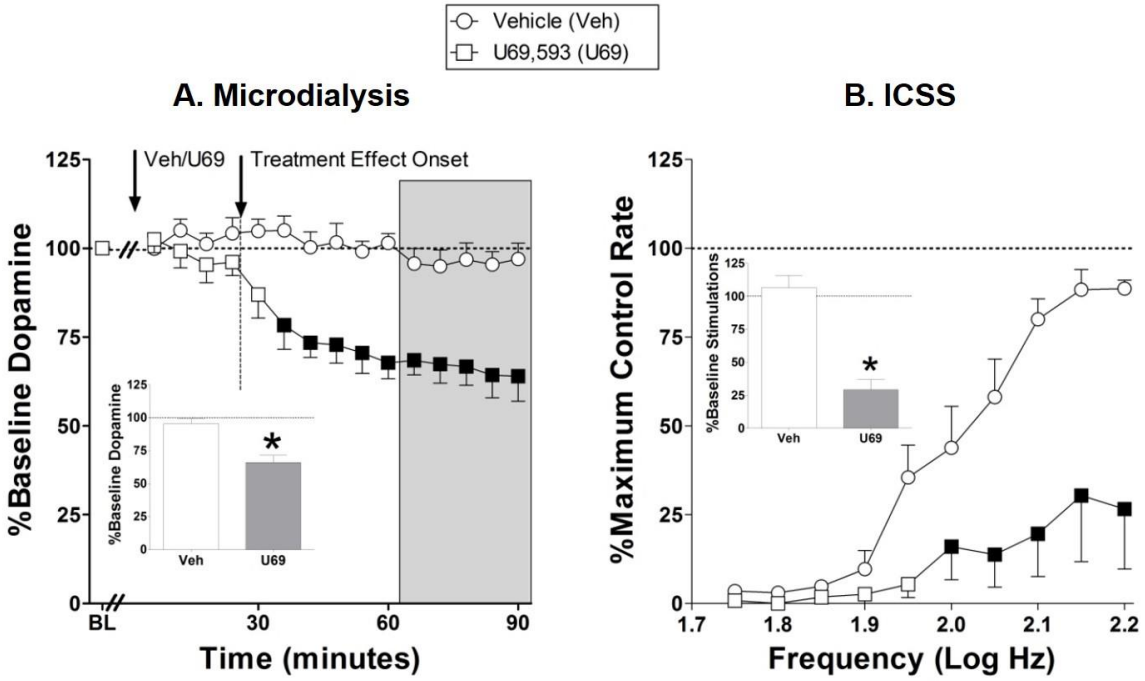
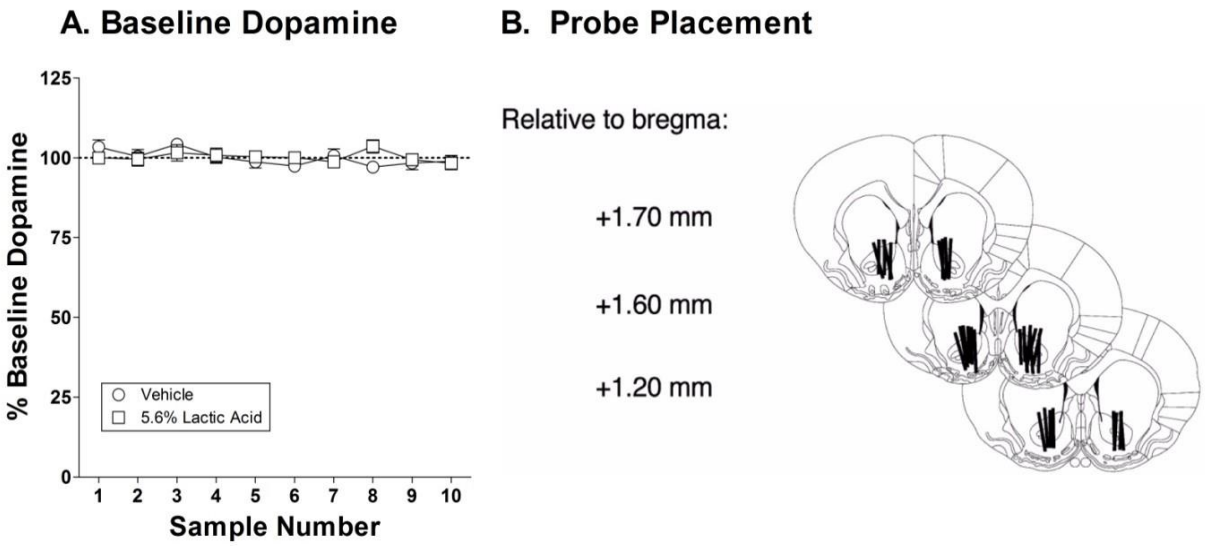

Vehicle (Veh)
U69,593 (U69)
A. Microdialysis
B. ICSS
Veh/U69
Treatment Effect Onset
%Baseline Dopamine
Time (minutes)
BL
30
60
90
%Maximum Control Rate
Frequency (Log Hz)
%Baseline Stimulations
Veh
U69
*

Figure II.7


A. Baseline Dopamine
% Baseline Dopamine
125
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10
Sample Number
Vehicle
5.6% Lactic Acid
B. Probe Placement
Relative to bregma:
+1.70 mm
+1.60 mm
+1.20 mm

Chapter III: Expression of chronic pain-depressed behavior and the role of endogenous kappa opioid system activation in rats

Published: Leitl, M.D., Potter, D.N., Cheng, K., Rice, K.C., Carlezon Jr, W.A., and Negus, S.S. (2014a). Sustained pain-related depression of behavior: effects of intraplantar formalin and complete freund's adjuvant on intracranial self-stimulation (ICSS) and endogenous kappa opioid biomarkers in rats. *Molecular Pain, 10:* 62.

Introduction

Preclinical procedures to evaluate pain and analgesia in laboratory animals play a key role in research on both neurobiology of pain and analgesic drug development (Negus et al., 2006; Mogil et al., 2010; Whiteside et al., 2013). Two common chemical stimuli for induction of sustained pain states in rodents are intraplantar administration of Complete Freund's Adjuvant (CFA) and formalin. CFA is a heat-killed bacterial suspension that elicits an immune response at the site of its injection. For example, CFA administration in the hindpaw of rats or mice produces paw edema (Brannen et al., 1975; Stein et al., 1988; Djouhri and Lawson, 1999) and increased local concentrations of inflammatory cytokines and trophic factors (Woolf et al., 1997). These and other inflammatory mediators contribute to peripheral and central sensitization of nociceptive neural pathways (Hylden et al., 1989; Ma and Woolf, 1996; Djouhri and Lawson, 2001), and this neural hypersensitivity correlates with a sustained behavioral hypersensitivity of withdrawal responses to mechanical or thermal stimuli (Stein et al., 1988; Hargreaves et al., 1988; Lin et al., 2007). These hypersensitive withdrawal responses often serve as a behavioral indicator of "pain."

Formalin, in contrast, is an aqueous solution of formaldehyde, a cell toxin that cross links proteins to disrupt dynamic protein interactions critical to cell viability. Formalin functions as an acute irritant, and when administered into the hindpaw of rodents, it elicits transient behaviors such as flinching and paw-licking (Tjølsen et al., 1992; Abbott et al., 1999; Fu et al., 2001; Lin et al., 2007). However, formalin also elicits a sustained inflammatory response characterized by edema and local release of inflammatory mediators (Lin et al., 2007). Moreover, formalin also damages or kills cells, including primary nociceptors and other sensory neurons, and as a result, formalin-induced changes in behavior also include a neuropathic component. For example, formalin injection into the hindpaw of rodents has been shown to produce general necrosis at the site of injection, increased expression of the neuropathy-related transduction factor ATF-3 in dorsal root ganglion cell bodies, and increased spinal microglial activation to an extent similar to that produced by other neuropathy models (Winter and McCarson, 2005; Lin et al., 2007; Berta et al., 2014). Consistent with this evidence for long-lasting tissue injury, intraplantar formalin also produces hypersensitivity to mechanical and thermal stimuli, and as with CFA, this hypersensitivity is sustained for days or weeks and often serves as a behavioral indicator of pain (Fu et al., 2001; Grace et al., 2014).

We have categorized behaviors such as hypersensitive paw withdrawal reflexes as "pain-stimulated behaviors," which are defined as behaviors that increase in rate, frequency or intensity after delivery of a pain stimulus (Negus et al., 2006; Negus et al., 2010a). However, pain states can also depress other behaviors, and treatment of pain-related behavioral depression is a common goal of human and veterinary medicine (Turk et al., 2003; Brown et al., 2009). Research on pain-related depression of behavior can be accomplished with procedures that measure "pain-depressed behaviors," which can be defined as behaviors that decrease in rate,

frequency or intensity after a pain stimulus (Negus et al., 2006; Negus et al., 2010a). For example, ICSS is a procedure in which subjects perform an operant behavior (e.g. press a lever) to receive pulses of electrical stimulation that activate the mesolimbic DA system, and ICSS has been used to examine effects of various manipulations on brain reward function (Wise, 1996; Carlezon and Chartoff, 2007a; Negus and Miller, 2014). Previous studies have reported relatively transient depression of ICSS by acute noxious stimuli including IP injection of dilute acid or paw incision (Do Carmo et al., 2009; Negus, 2013; Ewan and Martin, 2014). The goal of the present study was to compare effects of intraplantar CFA and formalin as more sustained pain stimuli on ICSS in rats. We hypothesized that both stimuli would produce sustained depression of ICSS similar to their shared ability to produce sustained thermal and mechanical hypersensitivity. Pain-related depression of ICSS was evaluated for its sensitivity to reversal by the mu opioid analgesic morphine. In addition, pain-related depression of ICSS was evaluated for its relationship to central biomarkers of the endogenous kappa opioid system and its sensitivity to the kappa antagonist norBNI, because some other stressors depress behavior by activating central kappa systems (Knoll and Carlezon, 2010; Bruchas et al., 2010).

Materials and Methods

Subjects

Studies were conducted in male Sprague-Dawley rats (Harlan, Frederick MD) with initial weights of 285 to 350 g. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM. Food and water were continuously available in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University

Institutional Animal Care and Use Committee and complied with the National Research Council (2011) Guide for the Care and Use of Laboratory Animals.

Noxious stimuli and Drugs

CFA was obtained from Sigma Aldrich (St. Louis, MO; Catalog #F5881). Formalin was obtained from Fisher Scientific (Waltham, MA; Catalog #305-510) and diluted in saline to final concentrations as described below. Rats were lightly restrained in a soft cloth for 100 ul bilateral injections administered into the plantar aspect of the left and right hind paws using a 27 gauge needle. Morphine sulfate (National Institute on Drug Abuse Drug Supply Program; Bethesda, MD) and norBNI 2HCl (synthesized by K. Cheng and K. Rice) were dissolved in saline for SC injection, and doses are expressed as the salt.

Assay of ICSS

The Surgery, Apparatus, and Training details of this study are the same as those reported previously [Chapter II].

Experiment 1: Comparison of CFA and formalin:

Once training was complete, baseline pre-injection ICSS was assessed for three consecutive days. Next, rats received bilateral intraplantar injections of CFA, 5% formalin, or saline. On the day of injection (Day 0), 3 "baseline" ICSS components were conducted before injection, and pairs of ICSS test components were conducted 1, 3, and 10 hr after injection. In addition, in the formalin and associated saline control groups, ICSS was also evaluated during five consecutive test components from 0-50 mins after injection, a time when formalin has been reported to elicit unconditioned flinching responses ("Phase I" and "Phase II" of the formalin

response; (Tjølsen et al., 1992; Abbott et al., 1999; Fu et al., 2001; Lin et al., 2007). On Days 1-7 after injection, ICSS was evaluated during three-component test sessions beginning at approximately 3 PM each day. Additionally, on days 1, 3 and 7, manipulations of the height of the ICSS response lever were introduced in a subset of 6 rats from each group as a potential "use-dependent" measure of injection effects on ICSS responding. Specifically, after testing with the standard low lever height (1.5 in above the floor), ICSS was re-determined with a medium lever height (2.75 in), and a high lever height (4.5 in), and these lever heights required increasingly vertical postures by the rat and increased weight bearing by the injected rear paws. Lever heights were presented in ascending order, with a 30-min time out between testing at each height.

ICSS was significantly depressed in the formalin-treated group on Day 7 after injection. To assess the sensitivity of formalin-induced depression of ICSS to treatment with an analgesic drug, an additional test with the mu opioid agonist morphine was conducted on Day 8 in the formalin and associated control groups. Following determination of baseline ICSS performance during three ICSS components as described above, cumulative doses of morphine (0.32-3.2 mg/kg SC) were administered at 60 min intervals, such that each dose increased the total, cumulative dose by 0.5 log units. Thirty minutes after each dose of morphine, a pair of ICSS test components was conducted.

Paw edema, body weight and mechanical sensitivity were measured in conjunction with ICSS in all rats in Experiment 1. To assess edema, dorsal-ventral thickness of the left hind paw was measured with electronic digital calipers (Traceable Calipers, Friendswood, TX) to the nearest 0.01 mm. Body weights were assessed using an electric scale (resolution 0.1 g). The von

Frey filament test was used to measure sensitivity to a punctate pressure stimulus. Rats were placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to acclimate for at least 20 min. Subsequently, von Frey filaments (0.4 - 15 g in approximate 0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar aspect of the left hind paw using the "up-down" method to determine log median withdrawal threshold (Chaplan et al., 1994). Paw thickness and body weight were assessed for each of three days before intraplantar injections and daily on Days 1-7 after intraplantar injection. Mechanical sensitivity was assessed for three days before injection, 6 hr after injection on Day 0, and on Days 3 and 7 after injection. All measurements were determined after daily ICSS sessions.

Experiment 2: Dose-dependence and persistence of formalin effects:

Twenty-four rats were trained in the ICSS procedure and divided into four separate groups of six each that received 0.5% bilateral formalin, 5% unilateral formalin in one paw + saline in the opposite paw, 5% bilateral formalin, or bilateral saline control. ICSS was evaluated daily for three days before injection and for 14 days after injection.

Experiment 3: Effects of norBNI on formalin-depressed ICSS:

Twelve rats were trained in the ICSS procedure and divided into two groups of six rats that received bilateral 5% formalin or bilateral saline control. ICSS was evaluated for three days before injection and for 14 days after injection. NorBNI (32 mg/kg SC) was administered immediately after ICSS testing on Day 7. Previous studies found that this dose of norBNI was

sufficient to block depression of ICSS produced by the kappa agonist U69,593 for at least three days (Leitl et al., 2014b).

Data analysis

The primary dependent measure for ICSS experiments was the total number of stimulations delivered across all 10 frequency trials of each component. The first ICSS component each day was considered to be a warm-up component, and data were discarded. Baseline ICSS in each subject was determined by averaging the number of stimulations per component during the second and third components across the three pre-injection baseline days (6 components total). Data collected after intraplantar injections for each subject were then normalized to these baselines using the equation % Baseline Stimulations per Component = (Stimulations per Test Component /Baseline) × 100.

An additional dependent measure was the reinforcement rate in stimulations/trial during each of the 10 frequency trials. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percentage of maximum control rate (%MCR) for that rat, with the maximum control rate defined as the mean of the maximal rates observed during any frequency trial of the second and third baseline components across the three pre-injection baseline days. Thus, %MCR values for each trial were calculated as (response rate during a frequency trial ÷ maximum control rate) × 100.

Data for ICSS, paw thickness, body weight and mechanical sensitivity were averaged across rats in each experimental condition and compared by two- or three-way ANOVA as

appropriate. For all analyses, a significant ANOVA was followed by the Holm-Sidak post-hoc test, and the criterion for significance was set a priori at $p < 0.05$.

Quantitative real-time reverse transcriptase polymerase chain reaction (studies conducted in collaboration with D.N. Potter and W.A. Carlezon Jr. at McLean Hospital, Harvard Medical School).

Twenty-four rats were used for qRT-PCR studies to assess CFA and formalin effects on endogenous PDYN and KOR mRNA in selected brain areas as described previously (Leitl et al., 2014b). Rats were treated with 100 ul bilateral intraplantar injections of saline, CFA, 0.5% formalin or 5% formalin (N = 6 per treatment), then euthanized seven days later by rapid decapitation. Brains were immediately extracted, rapidly frozen in -80°C isopentane, and stored at -80°C until analysis. Briefly, brains were sliced on a cryostat, and bilateral tissue punches were collected from VTA, NAaC and NAaS, CPu, and PFC. PDYN and KOR mRNA values were divided by the average values of the two internal controls (β-actin and Calnexin). Values are reported as percent saline controls calculated as (normalized saline and experimental means/normalized saline group mean for the corresponding time point) × 100. Data for PDYN and KOR mRNA were analyzed using two-way ANOVA with treatment and brain areas as the two factors.

Results

Figure III.1 shows effects of intraplantar saline, CFA or formalin on paw thickness, body weight and mechanical sensitivity. Intraplantar saline had no effect on paw thickness; however, both CFA and formalin produced significant and sustained increases in paw thickness (e.g. edema) within 24 hours, and these effects persisted for 7 days (Figure III.1 A, B). Body

weight increased significantly in the saline-control group for the CFA rats (Figure III.1 C), but this increase was small, and weight gain was not significant in the control group for the formalin rats (Figure III.1D). Administration of CFA, but not formalin, reduced body weight within 24 hours, and body weight in CFA rats remained below baseline for 7 days (Figure III.1C, D). Saline had no effect on mechanical sensitivity; however, both CFA and formalin produced significant and sustained decreases in mechanical thresholds for eliciting paw withdrawal within 6 hours, and this hypersensitivity persisted for 7 days (Figure III.1 E, F).

Figure III.2 Shows effects of intraplantar saline, CFA or formalin on ICSS. Prior to intraplantar injections, the mean ± SEM baseline number of stimulation per component across all stimulation frequencies was 264 ± 13, and the mean ± SEM maximum control rate (MCR) at any one frequency trial was 58.9 ± 2 stimulations per trial. Intraplantar saline treatment did not alter ICSS at any time. CFA produced weak and transient depression of ICSS. Specifically, at 1 and 3 hr after injection, ICSS in the CFA-treated rats was significantly lower than ICSS in the saline-treated controls (Figure III.2 A); however, ICSS in CFA- and saline-treated rats did not differ after 3 hr, and within the CFA group, ICSS after CFA treatment never differed significantly from baseline ICSS before CFA. Figure III.2 (C) shows full frequency-rate ICSS curves at selected times in the CFA-treated group. At baseline before CFA administration, electrical brain stimulation maintained a frequency-dependent increase in ICSS. In this analysis, ICSS was significantly decreased at several brain-stimulation frequencies after 1 hr but not after 7 days. In contrast to CFA, formalin produced a more robust and sustained depression of ICSS (Figure III.2 B). Relative to the saline control group, formalin significantly reduced ICSS during the first 24 hr after treatment and again on Day 3 and during Days 5-7 after treatment. Relative to the pre-formalin baseline within the formalin group, formalin significantly depressed ICSS during the

first 1 hr and again on Day 7 after treatment. Figure III.2 D shows full frequency-rate curves at selected times in the formalin treated group. Formalin significantly depressed ICSS at multiple frequencies both 1 hr and 7 days after treatment.

Figure III.3 Shows formalin effects on ICSS during the first 50 min after formalin administration. Formalin decreased ICSS throughout the first 50 min of testing, although the magnitude of this decrease was greatest from 20-50 min after treatment.

Figure III.4 Shows CFA and formalin effects on ICSS at different lever heights in a subset of six rats from each group on Days 1, 3 and 7 after intraplantar treatment. In general, ICSS decreased as lever height increased, and this effect was largest on Day 1. CFA failed to significantly alter ICSS at any lever height on any day, whereas formalin depressed ICSS across all lever heights and days.

Figure III.5 Shows effects of morphine on ICSS 8 days after treatment with intraplantar saline or formalin. In the saline-treated rats, cumulative dosing with 0.32-3.2 mg/kg morphine produced no significant effect on ICSS. Conversely, in the formalin-treated rats, baseline ICSS was depressed before morphine administration, and morphine produced a dose-dependent reversal of formalin-induced depression of ICSS. The lowest dose of 0.32 mg/kg morphine was sufficient to restore ICSS back to approximately baseline levels, and higher morphine doses produced a further facilitation of ICSS.

Figure III.6 compares ICSS performance over a 14-day period after treatment with bilateral saline, bilateral 0.5% formalin, unilateral 5% formalin in one paw + unilateral saline in the other paw, and bilateral 5% formalin. Prior to intraplantar injections, the mean ± SEM baseline number of stimulations per component across all stimulation frequencies was 274 ± 14, and the mean ± SEM MCR at any one frequency was 55.9 ± 3. As in the first experiment,

bilateral 5% formalin depressed ICSS relative to bilateral saline treatment, and in this second experiment, the formalin effect persisted for 14 days. Unilateral 5% formalin decreased ICSS relative to saline controls only on Days 1 and 6, and bilateral 0.5% formalin did not alter ICSS relative to the saline controls.

Figure III.7 Shows data to address the role of kappa opioid systems in mediating effects of CFA and formalin on ICSS. Neither CFA nor formalin significantly altered expression of PDYN or KOR mRNA in any brain area examined on Day 7 after intraplantar treatment (Figure III.5 A, B). Moreover, the kappa antagonist norBNI failed to alter ICSS in rats treated with intraplantar saline, and it also failed to block formalin-induced depression of ICSS in rats treated with intraplantar formalin (Figure III.V C-E). Data in Figure III.V5 (C-E) show results obtained on Day 8 after intraplantar injection and 22 hr after norBNI administration. Rats were also tested daily for another six days (Days 9-14 after intraplantar injection), and norBNI did not significantly alter either control ICSS or formalin-depressed ICSS on any day (data not shown).

Summary

This study compared effects of intraplantar CFA and formalin on a series of behavioral and physiological endpoints in rats. There were four main findings. First, consistent with previous studies, both CFA and formalin produced similar paw swelling and mechanical hypersensitivity. Second, CFA produced weak and transient depression of ICSS, whereas formalin produced a more robust and sustained depression of ICSS that lasted at least 14 days. Third, formalin-induced depression of ICSS was reversed by morphine doses that did not significantly alter ICSS in saline-treated rats, suggesting that formalin effects on ICSS can be interpreted as an example of pain-related and analgesic-reversible depression of behavior.

Finally, formalin-induced depression of ICSS was not associated with changes in central biomarkers for activation of endogenous kappa opioid systems, which have been implicated in depressive-like states in rodents, nor was it blocked by the kappa antagonist norBNI. These results suggest differential efficacy of sustained pain stimuli to depress brain reward function in rats as assessed with ICSS. Formalin-induced depression of ICSS does not appear to engage brain kappa opioid systems.

Figure Legends

Figure III.1 Effects of CFA, formalin, or respective controls on paw width, body weight and mechanical sensitivity. The abscissae for all panels is hours or days following bilateral intraplantar 100 ul injection of CFA (gray bars, Panels A, C, E), formalin (filled bars, Panels B, D, F), or respective saline controls (open bars, all panels). Bars above "BL" show baseline data before injection. Ordinates (Panels A, B): paw width in mm. Ordinates (Panels C, D): body weight in grams. Ordinates (Panels E, F): paw withdrawal threshold from von Frey filaments in grams (log scale). Dollar signs ($) indicate a significant within-group difference from the respective baseline, and asterisks (*) indicate a significant between-group difference at a given time point, as determined by a significant two-way Repeated Measures ANOVA followed by the Holm-Sidak post hoc test ($p < 0.05$). All points show mean ± SEM from 8 rats.

Figure III.2 Effects of CFA, formalin, or respective controls on ICSS. Panels A and B: Abscissae show hours or days following bilateral intraplantar 100 ul injection of CFA (gray bars, Panel A), formalin (filled bars, Panel B), or respective saline controls (open bars, both panels). Ordinates show ICSS rate expressed as total stimulations per component relative to pre-injection baseline. Dollar signs ($) indicate a significant within-group difference from the respective baseline, and asterisks (*) indicate a significant between-group difference at that time point.

Panels C and D show full frequency-rate ICSS curves for selected time points from A and B. Abscissae show frequency of electrical brain stimulation (Log Hz). Ordinates show ICSS rate expressed as percent maximum control rate (%MCR). Filled points indicate statistical significance of treatment effects relative to the pre-injection baseline. All statistical analyses were performed using two-way Repeated Measures ANOVA followed by the Holm-Sidak post hoc test ($p < 0.05$). All points show mean ± SEM from 8 rats.

Figure III.3 Shows effects of formalin (filled bars) or saline (open bars) on ICSS during the first 50 minutes of testing immediately following intraplantar administration. Abscissa: Time (in 10 minute bins) after intraplantar formalin or saline administration. Ordinates: ICSS rate expressed as total stimulations per component relative to baseline. Data were analyzed by two-way ANOVA followed by the Holm-Sidak post-hoc test ($p < 0.05$). All points show mean ± SEM from 8 rats. Statistical results are as follows. Significant main effect of treatment [$F(1, 14) = 13.141$; $p = 0.003$], significant main effect of time [$F(4,56) = 13.394$; $p < 0.001$], and a significant interaction of treatment x time [$F(4,56) = 3.928$; $p = 0.007$]. Dollar signs ($) indicate a significant within-group difference from the respective baseline, and asterisks (*) indicate a significant between-group difference at a given time point, as determined by a significant two-way ANOVA followed by the Holm-Sidak post hoc test ($p < 0.05$).

Figure III.4 Shows effects of CFA, filled gray bars in Panel A), formalin (filled bars in Panel B), or respective controls (open bars in both panels) on ICSS during lever height challenges. Abscissae show lever heights (low, middle or high) on days 1, 3 and 7 following treatment. Ordinates show ICSS rate expressed as total stimulations per component relative to baseline determined at the low lever height before intraplantar treatment. Data were analyzed by three-way ANOVA followed by the Holm-Sidak post-hoc test

($p < 0.05$). All points show mean ± SEM from 6 rats. Statistical results are as follows. Panel A. Significant main effect of day [$F(2,90) = 4.148$, $p = 0.019$], significant main effect of lever height [$F(2,90) = 9.097$, $p < 0.001$], but no main effect of CFA treatment [$F(1,90) = 0.121$, $p = 0.729$] and no significant interactions of day X lever height ($p = 0.785$), day X treatment ($p = 0.417$), lever height X treatment ($p = 0.696$), or day X lever height X treatment ($p = 0.803$). Panel B. Significant main effect of day [$F(2,90) = 4.669$, $p = 0.012$], significant main effect of lever height [$F(2,90) = 27.314$, $p < 0.001$], and a significant main effect of formalin treatment [$F(1,90) = 78.725$, $p < 0.001$], but no interactions of day X lever height ($p = 0.108$), day X treatment ($p = 0.154$), lever height X treatment ($p = 0.794$), or day X lever height X treatment ($p = 0.558$). Panels C-E show the posture of a rat responding at the low, medium and high lever height, respectively. Increases in lever height required increasingly erect postures and increased weight bearing on the hind paws.

Figure III.5 Effects of the mu opioid agonist morphine (0.32-3.2 mg/kg) on ICSS eight days after bilateral intraplantar saline or 5% formalin. Panels A and B: Abscissae show frequency of electrical brain stimulation (Log Hz) in rats that received intraplantar saline (A) or formalin (B). Ordinates show ICSS rate expressed as percent maximum control rate (%MCR). "BL" shows the baseline frequency-rate curve determined before intraplantar treatment, "0.0" shows the frequency-rate curve determined on Day 8 after intraplantar treatment but before morphine treatment. Filled points in panel B show significant morphine effects relative to "0.0." Panel C: The abscissa shows morphine dose in mg/kg in rats that received intraplantar saline (open bars) or formalin (filled bars). The ordinate shows ICSS rate expressed as total stimulations per component relative to baseline. Dollar signs ($) indicate a significant within-group difference from "0.0", and asterisks (*) indicate a significant between-group difference at

a given morphine dose. All statistical analyses were performed using a two-way Repeated Measures ANOVA followed by the Holm-Sidak post-hoc test ($p < 0.05$). All data show mean ± SEM from 8 rats per treatment.

Figure III.6 Formalin induced-depression of ICSS is dose and time related. Abscissa: Days after varying doses of intraplantar formalin or saline administration. Ordinate: ICSS rate expressed as percent baseline stimulations per component. Statistical analysis was performed using two-way Repeated Measures ANOVA followed by the Holm-Sidak post hoc test ($p < 0.05$). Dollar signs ($) indicate a significant within-group difference from the pre-injection baseline, and filled points indicate a significant between-group difference at that time point relative to saline treatment. All data show mean ± SEM from 6 rats per treatment.

Figure III.7 Role of the endogenous kappa opioid system in pain-related depression of ICSS. Panels A and B: Transcript levels of PDYN (A) or KOR (B) mRNA as measured by qRT-PCR in brain regions implicated in DA-ergic control of behavior. Abscissae: Brain area evaluated. Ordinates: Transcript levels expressed as "Fold-Induction" relative to saline controls. "ND" in Panel B signifies "Not Determined" due to low transcript levels below the level of detection in some rats. Panel C-D: Effects of the kappa antagonist norBNI (32 mg/kg) on ICSS in rats treated with intraplantar saline (C) or formalin (D). Abscissae show frequency of electrical brain stimulation (Log Hz). Ordinates show ICSS rate expressed as percent maximum control rate (%MCR). "BL" shows the frequency-rate curve determined on Day 7 after intraplantar saline or formalin and immediately before norBNI treatment. ICSS was then redetermined 24 hr after norBNI. Summary data are shown in Panel E, where the abscissa shows norBNI dose in mg/kg in rats that received intraplantar saline (open bars) or formalin (filled bars), and the ordinate shows ICSS rate expressed as total stimulations per component relative to

the pre-formalin baseline. The asterisk (*) indicates a significant between-group difference at a given dose. All data show mean ± SEM from 6 rats.

Figure III.1

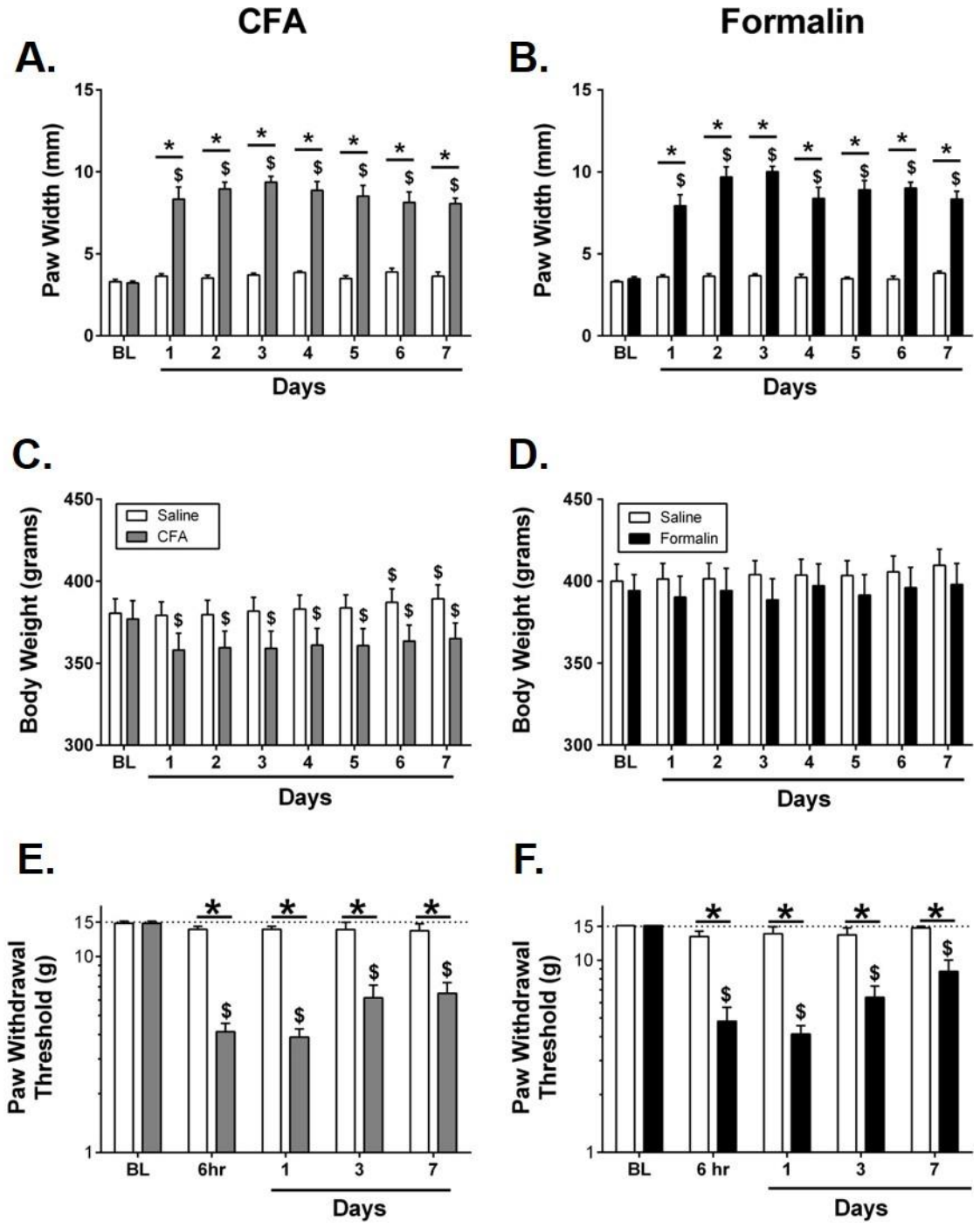

CFA
Formalin
A.
B.
Paw Width (mm)
Days
BL
C.
D.
Body Weight (grams)
Saline
CFA
Formalin
E.
F.
Paw Withdrawal Threshold (g)
6hr
6 hr

Figure III.2

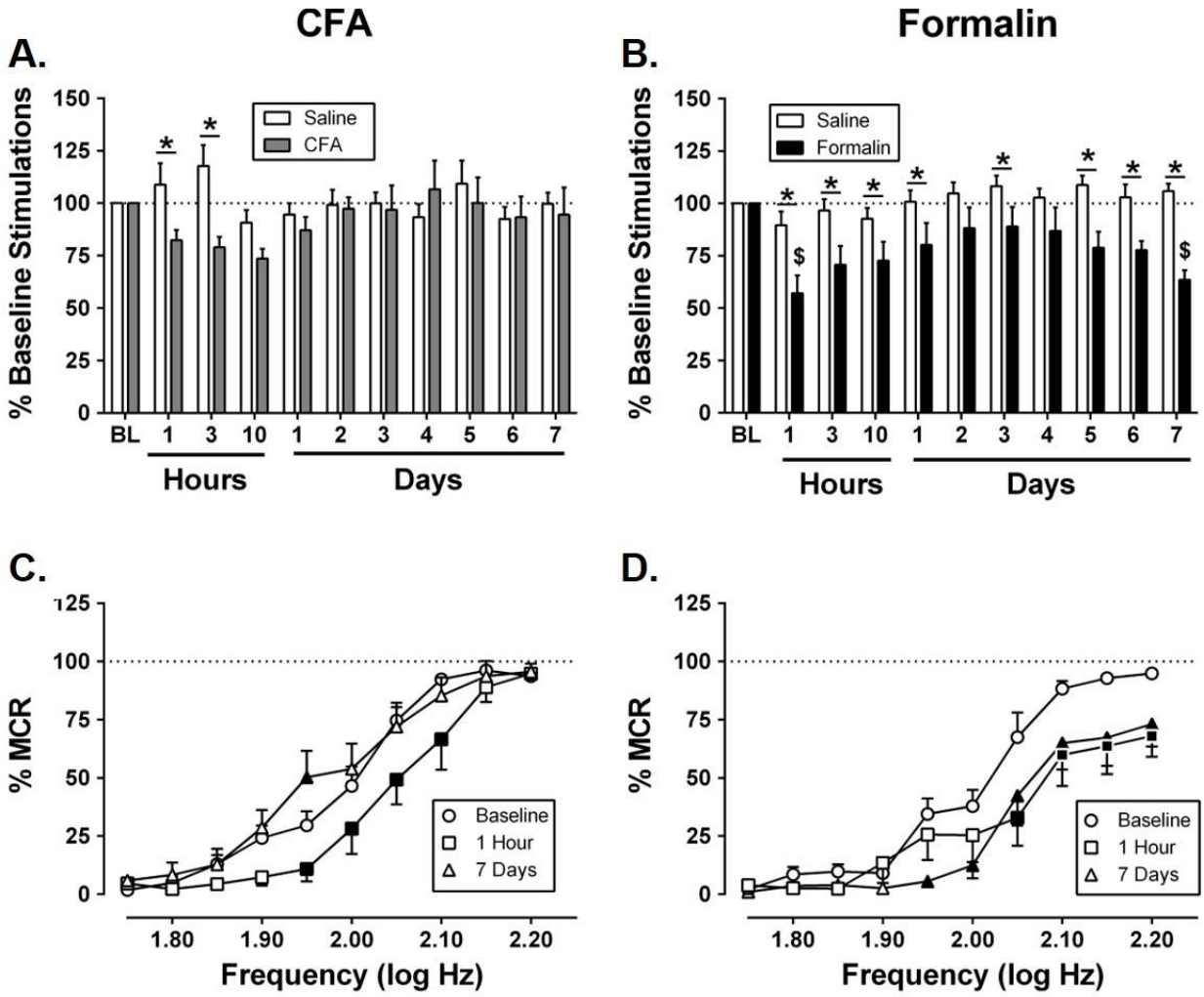

CFA
A.
% Baseline Stimulations
Saline
CFA
BL 1 3 10 1 2 3 4 5 6 7
Hours
Days
Formalin
B.
% Baseline Stimulations
Saline
Formalin
$
BL 1 3 10 1 2 3 4 5 6 7
Hours
Days
C.
% MCR
Baseline
1 Hour
7 Days
1.80 1.90 2.00 2.10 2.20
Frequency (log Hz)
D.
% MCR
Baseline
1 Hour
7 Days
1.80 1.90 2.00 2.10 2.20
Frequency (log Hz)

Figure III.3

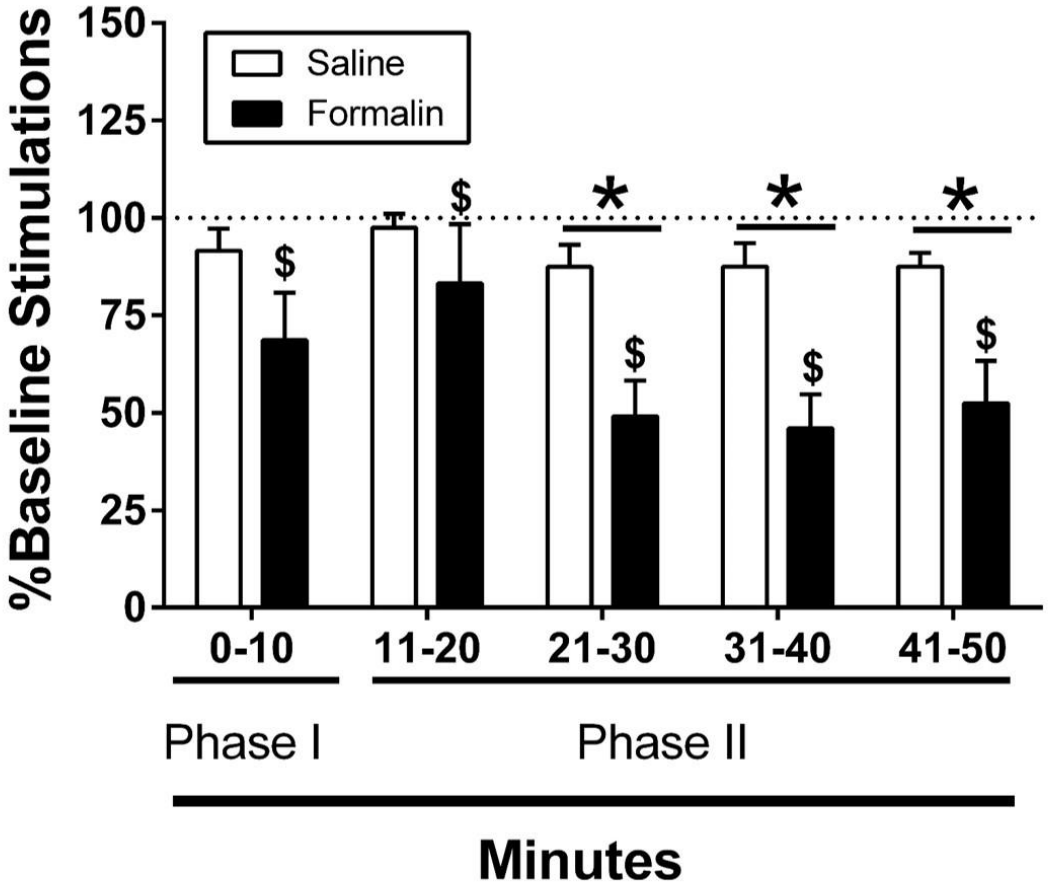

%Baseline Stimulations
150
125
100
75
50
25
0
Saline
Formalin
$
*
0-10
11-20
21-30
31-40
41-50
Phase I
Phase II
Minutes

Figure III.4

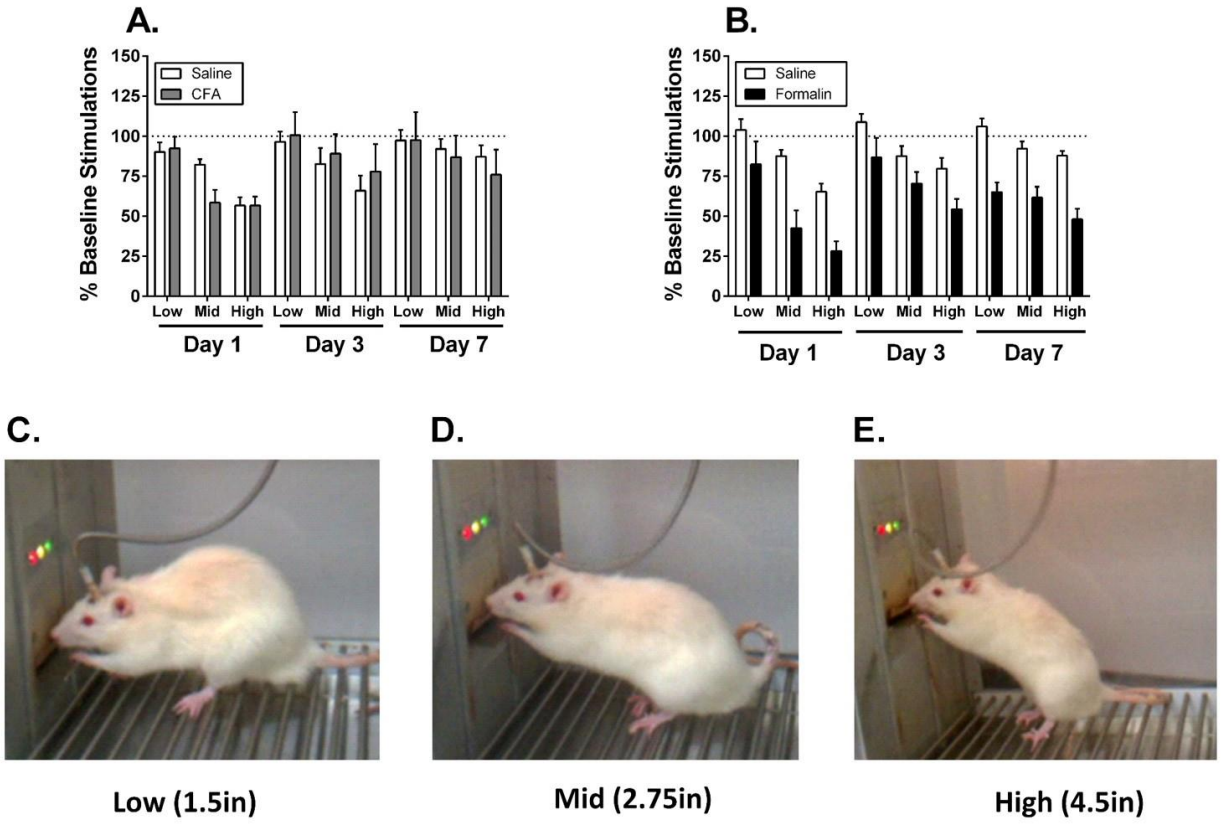

A.
% Baseline Stimulations
150
125
100
75
50
25
0
Saline
CFA
Low Mid High Low Mid High Low Mid High
Day 1
Day 3
Day 7
B.
% Baseline Stimulations
150
125
100
75
50
25
0
Saline
Formalin
Low Mid High Low Mid High Low Mid High
Day 1
Day 3
Day 7
C.
D.
E.
Low (1.5in)
Mid (2.75in)
High (4.5in)

Figure III.5

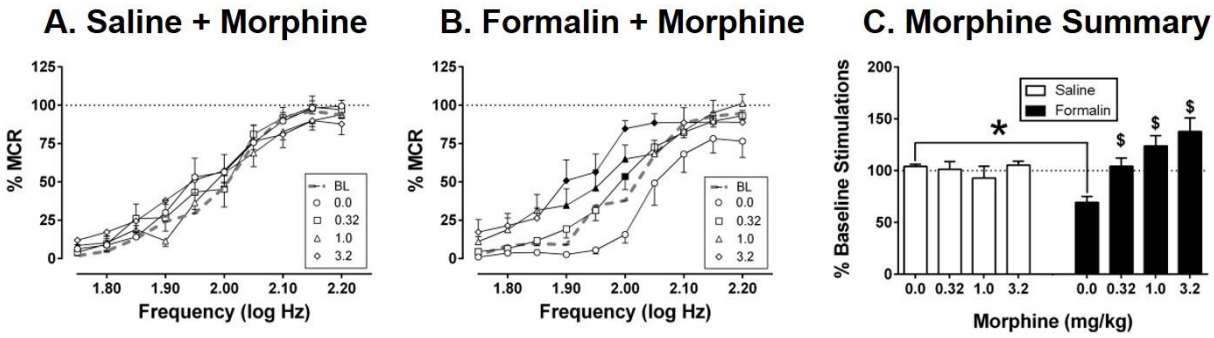

A. Saline + Morphine
B. Formalin + Morphine
C. Morphine Summary
% MCR
Frequency (log Hz)
BL
0.0
0.32
1.0
3.2
% Baseline Stimulations
Saline
Formalin
Morphine (mg/kg)

Figure III.6

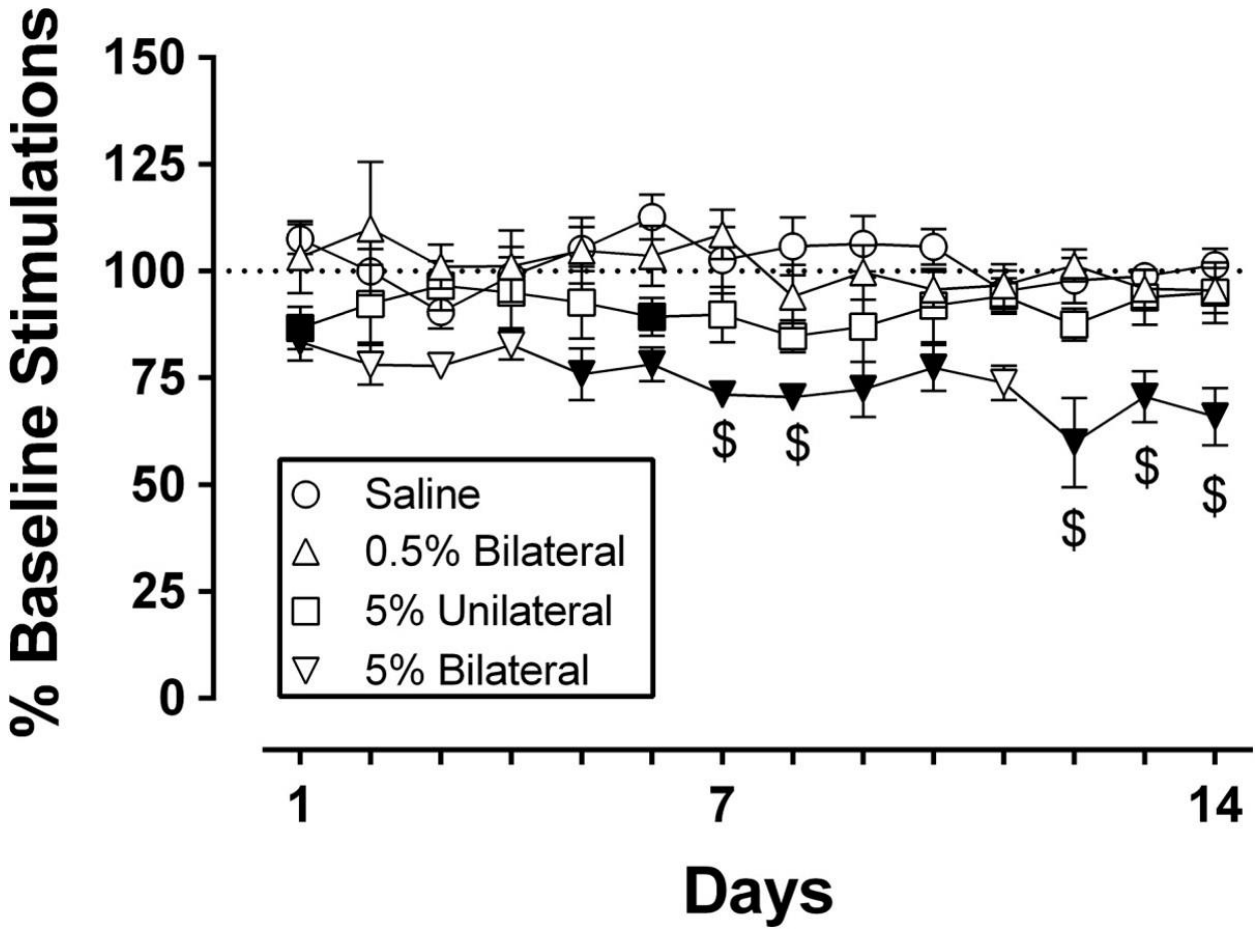
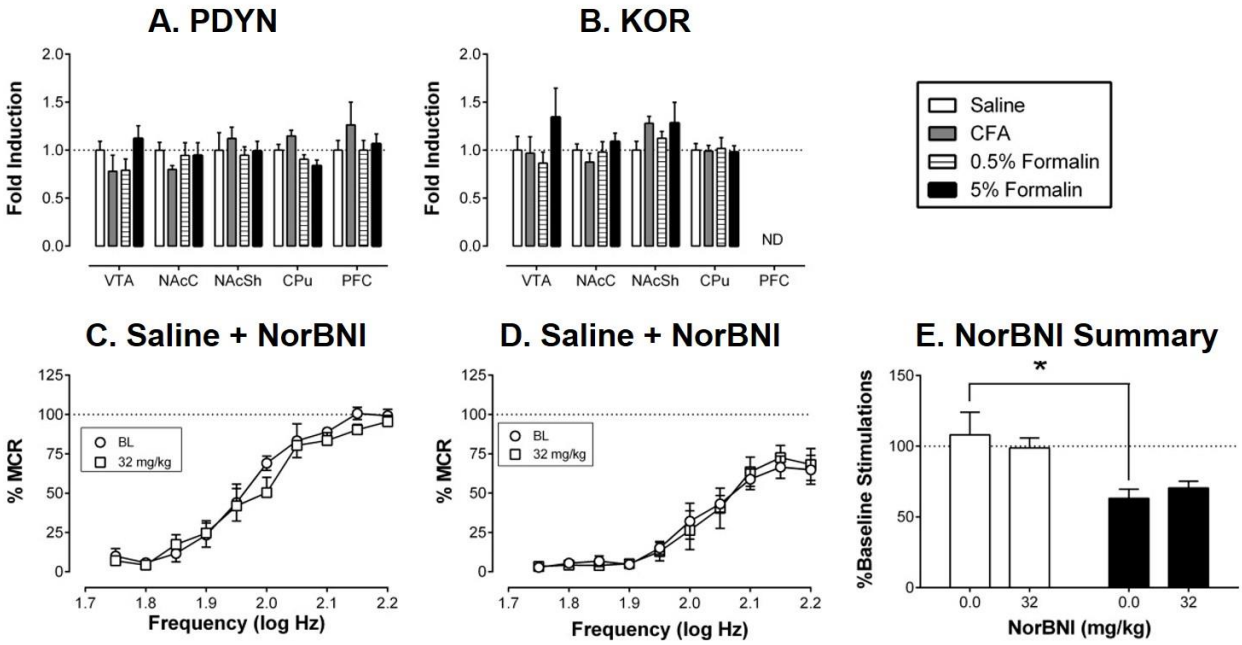

% Baseline Stimulations
150
125
100
75
50
25
0
Saline
0.5% Bilateral
5% Unilateral
5% Bilateral
$
$
$
$
$
1
7
14
Days

Figure III.7


A. PDYN
B. KOR
Fold Induction
2.0
1.5
1.0
0.5
0.0
VTA
NAcC
NAcSh
CPu
PFC
ND
Saline
CFA
0.5% Formalin
5% Formalin
C. Saline + NorBNI
D. Saline + NorBNI
% MCR
125
100
75
50
25
0
BL
32 mg/kg
1.7
1.8
1.9
2.0
2.1
2.2
Frequency (log Hz)
E. NorBNI Summary
%Baseline Stimulations
150
100
50
0
*
0.0
32
NorBNI (mg/kg)

Chapter IV: Pharmacological modulation of sustained pain-related depression of behavior: Effects of morphine, ketoprofen, bupropion, Δ9-tetrahydrocannabinol, and gabapentin on formalin-induced depression of intracranial self-stimulation (ICSS) in rats

Leitl MD and Negus SS. In preparation.

Introduction

Clinical pain is often associated with functional impairment and depression of behavior, and alleviation of pain-related depression of behavior is a common goal of treatment (Cleeland and Ryan, 1994; Dworkin et al., 2005). ICSS is a preclinical procedure in which operant behavior is maintained by delivery of electrical stimulation to brain reward areas, and pain-related depression of ICSS has served as one experimental tool for research on expression and treatment of pain-related depression of behavior in rats (Negus and Miller, 2014; Negus, 2013). ICSS in rats can be depressed by relatively transient pain stimuli including IP injection of dilute acid (Negus, 2013; Negus and Altarifi, 2013) and hindpaw incision (Ewan and Martin, 2014). Moreover, acid-induced depression of ICSS can be alleviated by treatment with clinically effective analgesics such as MOR agonists and nonsteroidal anti-inflammatory drugs, but not by treatment with other drug classes (e.g. centrally acting KOR agonists) that do not function as effective analgesics in humans despite producing apparent antinociception in many conventional preclinical pain assays (Negus et al., 2010; Negus et al., 2012). One implication of these findings is that preclinical assays of pain-related depression of ICSS or other behaviors may contribute to improved preclinical-to-clinical translation of results for candidate analgesics.

Although a need persists for safer and more effective analgesics to treat acute pain, there is a more pressing need to develop improved treatments for chronic pain in general and chronic neuropathic pain in particular (Institute of Medicine, 2011; Gilron et al., 2015; Kerstman et al., 2013) For example, one recent meta-analysis of neuropathic pain pharmacotherapies concluded that even the best pharmacotherapies available range from 4-10 in terms of patient "number needed to treat" (or NNT; Finnerup et al., 2015) to obtain a significant therapeutic effect in one patient. A common approach to modeling neuropathic pain in rodents involves strategies to injure sensory nerves innervating the rear paw to produce hypersensitive withdrawal responses to mechanical or thermal stimuli; however, a spinal nerve ligation injury sufficient to produce mechanical hypersensitivity failed to decrease ICSS in rats (Ewan and Martin, 2014), and this finding is consistent with other evidence to suggest a general absence of pain-related behavioral depression in common nerve injury models (Urban et al., 2011; LaCroix-Fralish et al., 2011). As an alternative to nerve injury models, formalin is an aqueous formulation of formaldehyde that cross links proteins to produce cell death, including neuropathy (Fu et al., 2001; Fu et al., 2000; Vierck et al., 2008), and we recently reported that bilateral intraplantar administration of dilute formalin produced not only mechanical hypersensitivity, but also a sustained pain-related depression of ICSS in rats (Leitl et al., 2014a). These results suggested that formalin-induced depression of ICSS in rats may serve as a useful procedure to evaluate drug effects on behavioral depression associated with sustained neuropathic pain.

The present study had two goals. First, we reported previously that acute morphine treatment produced a dose-dependent reversal of formalin-induced depression of ICSS (Leitl et al., 2014a). However, opioids and other pharmacotherapies for neuropathic pain are typically administered chronically, and changes in drug effects (e.g. tolerance to analgesic effects or to

undesirable side effects) can influence drug effectiveness and safety (Turk et al., 2003). Accordingly, one goal of this study was to assess potential changes in morphine effects during repeated treatment. Based on previous studies to evaluate effects of repeated morphine on acute acid-induced depression of ICSS (Altarifi and Negus, 2011), we hypothesized that repeated morphine would retain its antinociceptive efficacy, and that tolerance would develop to undesirable sedative effects. Second, we compared effects of repeated morphine to effects of repeated treatment with ketoprofen, bupropion, THC, and gabapentin. Ketoprofen is an NSAID analgesic that blocks acid-induced depression of ICSS (Negus et al., 2012), but NSAIDs are not effective against neuropathic pain (McQuay, 2007), and we hypothesized that it would be ineffective to block neuropathic pain-related depression of ICSS produced by formalin. Bupropion is a DA/NE uptake inhibitor used clinically as an antidepressant (Semenchuk et al., 2001). Bupropion blocks acid-induced depression of ICSS (Rosenberg et al., 2013), but bupropion and some other monoamine uptake inhibitors also have clinical efficacy to treat neuropathic pain (Finnerup et al., 2015; Semenchuk et al., 2001), and we hypothesized that bupropion would display sustained effectiveness to reverse formalin-induced depression of ICSS. THC, a cannabinoid receptor agonist and the principal psychoactive constituent of marijuana failed to block acid-induced depression of ICSS (Kwilasz and Negus, 2012), and although some studies have suggested effectiveness of THC to treat some forms of neuropathic pain (Phillips et al., 2010; Beaulieu and Ware, 2007; FASAM et al., 2005), a recent meta-analysis recommended against use of THC due to concerns over poor efficacy and unacceptable side effects (Finnerup et al., 2015). We hypothesized that THC would lack efficacy to reverse formalin-induced depression of ICSS. Gabapentin is an anticonvulsant that is considered a first line therapy for neuropathic pain, but clinical effectiveness is limited despite relatively strong

preclinical evidence (Finnerup et al., 2015; Chang et al., 2014; Kerstman et al., 2013), suggesting that over-reliance on pain-stimulated dependent measures may be contributing to the appearance of efficacy. For comparison with drug effects on formalin-induced depression of ICSS, effects of all drugs were also examined on the more conventional measure of mechanical hypersensitivity.

Materials and Methods

Subjects

Studies were conducted in male Sprague-Dawley rats (Harlan, Frederick MD) with initial weights of 285 to 350 g. Rats were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM. Food and water were continuously available in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council (2011) Guide for the Care and Use of Laboratory Animals.

Noxious Stimulus and Drugs

Formalin was obtained from Fisher Scientific (Waltham, MA; Catalog #305-510) and diluted in saline to obtain a 5% final concentration. Rats were lightly restrained in a soft cloth for 100µl bilateral injections administered into the plantar aspect of the left and right hind paws using a 27 gauge needle. Morphine sulfate (NIDA Drug Supply Program, Bethesda, MD) and bupropion HCl (Sigma Chemical, St. Louis, MO) were dissolved in sterile saline. Ketoprofen (Spectrum Chemical Co., New Brunswick, NJ), THC (NIDA Drug Supply Program) and gabapentin were prepared in a vehicle of ethanol, Emulphor EL-630 (Rhone-Poulenc; Princeton,

NJ), and sterile saline in a ratio of 1:1:18, respectively. For all drugs, doses are expressed as the drug forms named above.

Assay of ICSS. The Surgery, Apparatus, and Training details of this study are the same as those reported previously [Chapter II].

Experiment 1: Comparison of morphine, ketoprofen, bupropion, Δ9-tetrahydrocannabinol (THC), and gabapentin on ICSS:

We reported previously that bilateral intraplantar injections of 100 µl/paw 5% formalin was the lowest formalin treatment regimen to produce a stable, pain-related depression of ICSS, and this depression of ICSS was apparent from 7-14 days after formalin treatment (Leitl et al., 2014a). Accordingly, once stable ICSS was established, studies with each drug were conducted over a period of 14 days as illustrated in Figure IV.I. On Day 0, rats received bilateral intraplantar injections of 5% formalin or saline. On Days 1-6, no treatments were administered, and ICSS was evaluated daily during three-component sessions to monitor onset of formalin-induced depression of ICSS. On Days 7-13, drugs were administered and ICSS testing continued in three phases. First, on Day 7, a dose-ranging experiment was conducted using a cumulative dosing procedure. On these days, experimental sessions consisted of three daily-baseline components followed by three 60-min test periods. A dose of drug was administered at the beginning of each test period, and 30 min later, ICSS was evaluated during two ICSS test components. Each sequential dose increased the total cumulative dose by 0.5 or 1.0 log units, and dose ranges for each drug were as follows: morphine (0.32-3.2 mg/kg, SC), ketoprofen (0.1-10 mg/kg, IP), bupropion (3.2-32 mg/kg, IP), THC (0.32-3.2, IP), and gabapentin (3.2-32 mg/kg, IP). Doses, routes of administration, and pretreatment times were based on previous ICSS

studies with morphine, ketoprofen, bupropion, THC, and gabapentin (Altarifi et al., 2014; Kwilasz and Negus, 2012; Rosenberg et al., 2013; Ruyang et al., 2015). Next, on Days 8-13, the effects of repeated daily dosing with a single drug dose were examined. Experimental sessions consisted of three daily-baseline components followed immediately by administration of a single dose of test drug and then 30 min later by two ICSS test components. The dose of drug administered on Days 8-13 was selected based on results of the Day 7 dose-ranging study as discussed below in Results. Finally, on Day 14, the cumulative dose-effect curve was re-determined using procedures identical to those on Day 7 to assess changes in drug effects associated with repeated treatment.

Data analysis. The primary dependent measure for ICSS experiments was the total number of stimulations delivered across all 10 frequency trials of each component. The first ICSS component each day was considered to be a warm-up component, and data were discarded. A "Pretreatment Baseline" measure of ICSS in each subject was determined by averaging the number of stimulations per component during the second and third components across the three baseline days before intraplantar formalin/saline treatments (6 components total; see Figure IV.I). Daily-baseline data and drug-test data collected after intraplantar injections for each subject were then normalized to the Pretreatment Baseline using an equation to calculate % Pretreatment Baseline Stimulations per Component. For daily-baseline components, data from the first component were discarded, and data from the second and third components on each day were first expressed as (Stimulations per Daily-Baseline Component /Pretreatment Baseline) x 100, and then averaged across components. For drug-test components, data for each of the two test component after each drug dose were expressed as (Stimulations per Drug-Test Component/Pretreatment Baseline) x 100, then averaged across components.

An additional dependent measure was the reinforcement rate in stimulations per trial during each of the 10 frequency trials of each component. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percentage of maximum control rate (%MCR) for that rat, with the maximum control rate defined as the mean of the maximal rates observed during any frequency trial of the second and third baseline components across the three Pretreatment Baseline days. Thus, %MCR values for each trial were calculated as (response rate during a frequency trial ÷ maximum control rate) × 100.

ICSS data were averaged across rats in each experimental condition and compared by one or two-way ANOVA, as appropriate. A significant ANOVA was followed by either a Dunnett's post-hoc test (one-way ANOVA) or a Holm-Sidak post-hoc test (two-way ANOVA), and the criterion for significance was set a priori at $p < 0.05$.

Experiment 2: Comparison of morphine, ketoprofen, bupropion, Δ9-tetrahydrocannabinol, and gabapentin on paw withdrawal from mechanical stimulation:

To provide a comparison with drug effects on formalin-induced depression of ICSS, separate groups of rats were used to assess drug effects on formalin-induced mechanical allodynia. Specifically, the von Frey filament test was used to measure sensitivity to a punctate pressure stimulus, as previously described (Leitl et al., 2014a). Briefly, rats were placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to acclimate for at least 20 min. Subsequently, von Frey filaments (0.4 - 15 g in approximate 0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar aspect of the left hind paw using the "up-down" method to determine log median withdrawal threshold (Chaplan et al., 1994). Thresholds were determined before intraplantar injection of formalin or

saline and again 7 days after intraplantar treatments. On day 7, thresholds were determined five times, once before any further treatment, once after treatment with drug vehicle, and three additional times after treatment with each of three cumulative doses of the test drug. The dose ranges and dose intervals for cumulative dosing were identical to those used on day 7 of ICSS studies.

Data analysis. The primary dependent measure for von Frey experiments was log median withdrawal threshold (Chaplan et al., 1994). These values were averaged across rats for each drug dose, and data for each drug dose were compared to the respective vehicle using one-way ANOVA followed by Dunnett post hoc test to compare paw withdrawal thresholds after each dose to the paw withdrawal thresholds after drug vehicle ($p<0.05$).

Results

Formalin-induced depression of ICSS. For all rats used in the study, the mean±SEM Pretreatment Baseline number of stimulations per component was 319.5±9.7, and the mean±SEM Maximum Control Rate (MCR) was 52.9±0.3 stimulations per trial. Prior to intraplantar treatment, pretreatment baseline rates of ICSS did not differ between groups that subsequently received either saline or formalin (data not shown). Figure IV.1 shows that bilateral injection of intraplantar formalin depressed ICSS by day 7 relative to ICSS in saline-treated rats. Specifically, formalin produced a rightward and downward shift in the ICSS frequency-rate curve (Figure IV.1B) and a decrease in the total numbers of stimulations per component delivered across all brain-stimulation frequencies (Figure IV.1C). Similar formalin effects were observed in each group of rats used to study test drugs, and formalin-induced depression of baseline ICSS was sustained throughout the subsequent period of drug testing

(Figures IV.2-4). This formalin-induced depression of ICSS served an example of sustained pain-related depression of behavior, and drugs were evaluated for their effectiveness to reverse formalin effects.

Effects of Morphine. Figure IV.2 shows effects of morphine on ICSS in saline-treated rats (Figure IV.IIA-C) and formalin-treated rats (Figure IV.2D-F). On day 7 after intraplantar saline treatment, cumulative doses of 0.32 and 1.0 mg/kg morphine did not alter ICSS, and 3.2 mg/kg morphine significantly depressed ICSS (Figure IV.2A). A dose of 1.0 mg/kg morphine was selected for daily treatments on days 8-13 after intraplantar saline (see below for rationale), and this dose did not alter ICSS on any day (Figure IV.2B). On day 14 after intraplantar saline, cumulative doses of 0.32-3.2 mg/kg morphine had no significant effect on ICSS (Figure IV.2C), indicating tolerance to the initial rate-decreasing effects of 3.2 mg/kg morphine observed on day 7.

On day 7 after intraplantar formalin treatment, baseline ICSS was depressed, and cumulative morphine reversed this formalin-induced depression of ICSS with an inverted-U shaped dose-effect curve (Figure IV.2D). Significant reversal was obtained with 1.0 mg/kg morphine, so this dose was used for repeated daily treatments on days 8-13 after intraplantar formalin. Two-way ANOVA during this treatment period indicated a significant main effect of morphine to alleviate formalin-induced depression of ICSS (Figure IV.2E). On day 14 after intraplantar formalin, cumulative morphine produced a dose-dependent reversal of formalin-induced depression of ICSS, with significant effects produced by 1.0 and 3.2 mg/kg morphine (Figure IV.2F).

Effects of Ketoprofen. Figure IV.3 shows effects of ketoprofen on ICSS in saline-treated rats (Figure IV.3A-C) and formalin-treated rats (Figure IV.3D-F). On day 7 after intraplantar saline treatment, cumulative doses of 0.1, 1.0, and 10 mg/kg ketoprofen did not alter ICSS. A dose of 10 mg/kg ketoprofen was selected for daily treatments on days 8-13 after intraplantar saline (see below for rationale), and this dose did not alter ICSS on any day (Figure IV.3B). On day 14 after intraplantar saline, cumulative doses of 0.1, 1.0, and 10 mg/kg ketoprofen did not alter ICSS (Figure IV.3C).

On day 7 after intraplantar formalin treatment, baseline ICSS was depressed, and cumulative ketoprofen doses up to 10 mg/kg did not reverse this formalin-induced depression of ICSS (Figure IV.3D). Because no ketoprofen dose altered ICSS, and higher doses may produce gastrointestinal toxicity in rodents (Lamon et al., 2008;de la Lastra et al., 2002), the dose of 10 mg/kg was selected for repeated daily treatments on days 8-13 after intraplantar formalin, and two-way ANOVA during this treatment period did not indicate a significant main effect of ketoprofen to alleviate formalin-induced depression of ICSS (Figure IV.3E). On day 14 after intraplantar formalin, cumulative doses of 0.1, 1.0, and 10 mg/kg ketoprofen did not alter ICSS (Figure IV.IIIF).

Effects of Bupropion. Figure IV.4 shows effects of bupropion on ICSS in saline-treated rats (Figure IV.4A-C) and formalin-treated rats (Figure IV.4D-F). On day 7 after intraplantar saline treatment, cumulative doses of 3.2 and 10 mg/kg bupropion did not alter ICSS, and 32 mg/kg bupropion significantly facilitated ICSS (Figure IV.4A). A dose of 3.2 mg/kg bupropion was selected for daily treatments on days 8-13 after intraplantar saline (see below for rationale), and this dose did not alter ICSS on any day (Figure IV.4B). On day 14 after intraplantar saline, cumulative doses of 3.2 and 10 mg/kg bupropion had no significant effect on ICSS, but 32 mg/kg

bupropion again significantly facilitated ICSS (Figure IV.4C), indicating a lack of sensitization or tolerance to the initial effects of bupropion observed on day 7.

On day 7 after intraplantar formalin treatment, baseline ICSS was depressed, and cumulative bupropion reversed this formalin-induced depression of ICSS in a dose-dependent matter (Figure IV.4D). Significant reversal was obtained with 10 and 32 mg/kg bupropion, but 3.2 mg/kg bupropion increased mean ICSS levels back to approximately 100% of the pre-formalin baseline, and the lack of statistical significance was due in part to high variability in effects of 32 mg/kg bupropion (e.g. 3.2 mg/kg bupropion did significantly increase ICSS relative to the daily baseline when evaluated by t-test, $p<0.05$). Accordingly, 3.2 mg/kg bupropion was used for repeated daily treatments on days 8-13 after intraplantar formalin, and two-way ANOVA during this treatment period indicated a significant main effect of bupropion to alleviate formalin-induced depression of ICSS (Figure IV.4E). On day 14 after intraplantar formalin, cumulative bupropion produced a dose-dependent reversal of formalin-induced depression of ICSS, with significant effects again produced by 10 and 32 mg/kg bupropion (Figure IV.4F). As on day 7, effects of 3.2 mg/kg bupropion were not statistically significant, but mean ICSS levels were restored to approximate baseline levels.

Effects of THC. Figure IV.5 shows effects of THC on ICSS in saline-treated rats (Figure IV.5A-C) and formalin-treated rats (Figure IV.5D-F). On day 7 after intraplantar saline treatment, cumulative doses of 0.32 and 1.0 mg/kg THC did not alter ICSS, and 3.2 mg/kg THC significantly depressed ICSS (Figure IV.5A). A dose of 1.0 mg/kg THC was selected for daily treatments on days 8-13 after intraplantar saline (see below for rationale), and this dose did not significantly alter ICSS during repeated treatment (Figure IV.5B). On day 14 after intraplantar saline, cumulative doses of 0.32, and 1.0 mg/kg THC did not alter ICSS, and 3.2 mg/kg again

significantly depressed ICSS (Figure IV.5C), indicating a lack of tolerance to the initial rate-decreasing effects of 3.2 mg/kg THC observed on day 7.

On day 7 after intraplantar formalin treatment, baseline ICSS was depressed, and cumulative THC produced a dose-dependent exacerbation of formalin-induced depression of ICSS (Figure IV.5D). Because none of the doses of THC tested alleviated formalin effects, an intermediate dose of 1.0 mg/kg THC was evaluated on days 8-13 to assess the potential for repeated treatment to produce tolerance to its rate-decreasing effects and unmask a reversal of formalin-induced depression of ICSS. However, two-way ANOVA during this treatment period did not indicate a significant main effect of THC (Figure IV.5E), and on day 14, cumulative THC again only exacerbated formalin-induced depression of ICSS (Figure IV.5F). A dose of 1.0 mg/kg THC, which significantly decreased ICSS on day 7 in the formalin-treated rats, did not significantly alter ICSS on Day 14, suggesting tolerance to the rate-decreasing effects of this THC dose.

Effects of Gabapentin. Figure IV.6 shows effects of gabapentin on ICSS in saline-treated rats (Figure IV.6A-C) and formalin-treated rats (Figure IV.6D-F). On day 7 after intraplantar saline treatment, cumulative doses of 3.2 and 10 mg/kg gabapentin did not alter ICSS, and 32 mg/kg gabapentin significantly depressed ICSS (Figure IV.6A). A dose of 32 mg/kg gabapentin was selected for daily treatments on days 8-13 after intraplantar saline (see below for rationale), and two-way ANOVA during this treatment period indicated a significant main effect of gabapentin, whereby gabapentin administration exacerbated or further decreased formalin-induced depression of ICSS (Figure IV.6B). On day 14 after intraplantar saline, cumulative doses of 3.2 and 10 mg/kg gabapentin did not alter ICSS, and 32 mg/kg significantly depressed ICSS

(Figure IV.6C), indicating a lack of tolerance to the initial rate-decreasing effects of 32 mg/kg gabapentin observed on day 7, and observed throughout the study.

On day 7 after intraplantar formalin treatment, baseline ICSS was depressed, and cumulative gabapentin produced a dose-dependent exacerbation of acid-induced depression of ICSS (Figure IV.6D). No significant reversal was obtained with any gabapentin dose, so the highest dose of 32 mg/kg was evaluated on days 8-13 to assess the potential for repeated treatment with this dose to produce tolerance to its rate-decreasing effects and thereby unmask a reversal of formalin-induced depression of ICSS. Two-way ANOVA during this treatment period revealed a significant main effect of gabapentin treatment, but this main effect was further depression of ICSS rather than a reversal of formalin-induced depression of ICSS (Figure IV.6E). On day 14 after intraplantar formalin, cumulative gabapentin again produced a dose-dependent exacerbation of formalin-induced depression of ICSS, with significant effects produced by all gabapentin doses (Figure IV.6F).

Drug effects on mechanical allodynia. Figure IV.7 shows that paw-withdrawal thresholds to mechanical stimulation were significantly reduced 7 days after formalin injection relative to pre-formalin values (IV.7A). Morphine, bupropion, THC, and gabapentin produced reversal of formalin-induced mechanical allodynia in a dose-dependent manner (IV.7B. Ketoprofen, in contrast, failed to reverse formalin-induced mechanical allodynia at any dose tested (IV.7B).

Summary

This study evaluated effects of repeated treatment with morphine, ketoprofen, bupropion, THC, and gabapentin on sustained pain-related depression of ICSS produced by intraplantar

formalin injection. For comparison, acute drug effects were also examined on the more conventional endpoint of formalin-induced mechanical allodynia using the von Frey assay. There were three main findings. First, in agreement with previous studies, intraplantar formalin produced both mechanical allodynia and sustained depression of ICSS. Second, morphine produced a dose-dependent reversal of both formalin-induced mechanical allodynia and formalin-induced depression of ICSS, and morphine antinociception in the assay of formalin-depressed ICSS was sustained during repeated treatment. Third, the DA/NE uptake inhibitor bupropion also blocked formalin-induced mechanical allodynia and produced a sustained reversal of formalin-induced depression of ICSS; however, ketoprofen was not effective to reverse either formalin effect, while THC and gabapentin were effective in reversing formalin-induced mechanical allodynia but not formalin-induced depression of ICSS. These results illustrate a range of potential effect profiles and provide further evidence to suggest that evaluation of drug effects on pain-related depression of ICSS may both (a) differ from drug effects on more conventional endpoints in preclinical pain assays, and (b) contribute new insights to preclinical evaluation of candidate analgesic drugs.

Figure Legends

IV.1 Effects of bilateral intraplantar treatment with saline or 5% formalin on ICSS for all rats used in ICSS studies. (A) Panel A shows the experimental timeline for behavioral testing, intraplantar treatments with formalin or saline, and drug treatments. (B) Panel B shows full frequency-rate curves determined on day 7 after saline or formalin treatment and before initiation of drug treatments. Abscissa: frequency of electrical brain stimulation (Log Hz). Ordinate: ICSS rate expressed as percent maximum control rate (%MCR). Two-way ANOVA indicated significant main effects of treatment [$F(1,35)=27.42$; $p<0.001$] and frequency [$F(9,207)=307.1$; $p<0.001$)], and a significant interaction [$F(9,207)=5.879$; $p<0.001$)]. Filled points indicate a significant between-group effect of treatment at a given brain-stimulation frequency (Holm-Sidak post hoc test, $p<0.05$). (C) Panel B shows summary data for the total number of stimulations delivered across all brain-stimulation frequencies on day 7 after saline or formalin treatment. Abscissa: Intraplantar treatment. Ordinate: total number of stimulations per component, expressed as a percentage of the pretreatment baseline. The asterisk indicates a significant difference between groups as determine by t-test ($t=5.910$; $p<0.001$). All points and bars show mean ± SEM from 36 rats.

IV.2 Effects of the mu opioid agonist morphine on ICSS 7-14 days after bilateral intraplantar saline (A-C) or 5% formalin (D-F). Panels A, C, D and F show effects of cumulative morphine (0.32-3.2 mg/kg) administered on day 7 (A,D) or day 14 (C,F) after intraplantar treatment. Abscissae: dose morphine in mg/kg. Baseline (BL) ICSS determined before morphine treatment is also shown in each panel. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. Asterisks indicate significantly different from the daily baseline as determined by a significant one-way ANOVA followed by

the Dunnett post hoc test ($p<0.05$; see ANOVA results below). Panels B and E show effects of 1.0 mg/kg morphine administered on days 8-13 after intraplantar treatment. Abscissae: day after intraplantar treatment. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. For each day, data are shown for ICSS before morphine administration (Daily Baseline) and after morphine administration (+1.0 morphine). The p value for the main effect of morphine treatment is shown in each panel (see below for full 2-way ANOVA results). ANOVA results for each panel were as follows: (A) significant effect of morphine dose [$F(3,15)=5.006$; $p=0.013$]; (B) no main effect of morphine [$F(1,5)=1.789$; $p=0.239$] or day [$F(5,25)=1.426$; $p=0.250$)], and no interaction [$F(5,25)=0.3102$; $p=0.902$]; (C) no significant effect of morphine dose [$F(3,15)=1.775$; $p=0.195$]; (D) significant effect of dose [$F(3,15)=9.994$; $p<0.001$]; (E) significant main effect of morphine [$F(1,5)=15.56$; $p=0.011$] but not day [$F(5,25)=1.106$; $p=0.382$], and no interaction [$F(5,25)=0.5758$; $p=0.718$]; (F) significant effect of dose [$F(3,15)=4.450$; $p=0.020$]. All points show mean ± SEM from 6 rats.

IV.3 Effects of the nonsteroidal anti-inflammatory drug ketoprofen on ICSS 7-14 days after bilateral intraplantar saline (A-C) or 5% formalin (D-F). Panels A, C, D and F show effects of cumulative ketoprofen (0.1-10 mg/kg) administered on day 7 (A,D) or day 14 (C,F) after intraplantar treatment. Abscissae: dose of ketoprofen in mg/kg. Baseline (BL) ICSS determined before ketoprofen treatment is also shown in each panel. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. Panels B and E show effects of 10 mg/kg ketoprofen administered on days 8-13 after intraplantar treatment. Abscissae: day after intraplantar treatment. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. For each day, data are shown for ICSS before ketoprofen administration (Daily Baseline) and after ketoprofen administration (+10.0 ketoprofen). The p

value for the main effect of ketoprofen treatment is shown in each panel (see below for full 2-way ANOVA results). ANOVA results for each panel were as follows: (A) no significant effect of dose [$F(3,15)=0.1368$; $p=0.937$]; (B) no main effect of ketoprofen [$F(1,5)=0.07948$; $p=0.789$] or day [$F(5,25)=0.2518$; $p=0.935$], and no interaction [$F(5,25)=0.9778$; $p=0.451$]; (C) no significant effect of dose [$F(3,15)=0.4254$; $p=0.425$]; (D) no significant effect of dose [$F(3,15)=0.164$; $p=0.164$]; (E) no significant main effect of ketoprofen [$F(1,5)=0.3149$; $p=0.5999$] or day [$F(5,25)=0.3279$; $p=0.891$], and no interaction [$F(5,25)=0.4982$; $p=0.775$]; (F) no significant effect of dose [$F(3,15)=1.815$; $p=0.188$]. All points show mean ± SEM from 6 rats.

IV.4 Effects of the DA uptake inhibitor bupropion on ICSS 7-14 days after bilateral intraplantar saline (A-C) or 5% formalin (D-F). Panels A, C, D and F show effects of cumulative bupropion (3.2-32 mg/kg) administered on day 7 (A,D) or day 14 (C,F) after intraplantar treatment. Abscissae: dose of bupropion in mg/kg. Baseline (BL) ICSS determined before bupropion treatment is also shown in each panel. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. Asterisks indicate significantly different from the daily baseline as determined by a significant one-way ANOVA followed by the Dunnett post hoc test ($p<0.05$; see ANOVA results below). Panels B and E show effects of 3.2 mg/kg bupropion administered on days 8-13 after intraplantar treatment. Abscissae: day after intraplantar treatment. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. For each day, data are shown for ICSS before bupriopion administration (Daily Baseline) and after bupropion administration (+3.2 bupropion). The p value for the main effect of bupropion treatment is shown in each panel (see below for full 2-way ANOVA results). ANOVA results for each panel were as follows: (A) significant effect of dose [$F(3,15)=6.088$; $p=0.006$]; (B) no main effect of bupropion [$F(1,5)=0.2095$; $p=0.666$] or day

[F(5,25)=0.8404; p=0.534], but a significant interaction [F(5,25)=5.454; p=0.002]; (C) significant effect of dose [F(3,15)=9.314; p=0.001]; (D) significant effect of dose [F(3,15)=7.151; p=0.003], (E) significant main effect of bupropion [F(1,5)=14.36; p=0.013] but not day [F(5,25)=0.2447; p=0.939], and no interaction [F(5,25)=1.773; p=0.167]; (F) significant effect of dose [F(3,15)=0.0029; p=0.003]. All points show mean ± SEM from 6 rats.

IV.5 Effects of the cannabinoid receptor agonist THC on ICSS 7-14 days after bilateral intraplantar saline (A-C) or 5% formalin (D-F). Panels A, C, D and F show effects of cumulative THC (0.32-3.2 mg/kg) administered on day 7 (A,D) or day 14 (C,F) after intraplantar treatment. Abscissae: dose of THC in mg/kg. Baseline (BL) ICSS determined before THC treatment is also shown in each panel. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. Asterisks indicate significantly different from the daily baseline as determined by a significant one-way ANOVA followed by the Dunnett post hoc test ($p<0.05$; see ANOVA results below). Panels B and E show effects of 1.0 mg/kg THC administered on days 8-13 after intraplantar treatment. Abscissae: day after intraplantar treatment. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. For each day, data are shown for ICSS before THC administration (Daily Baseline) and after THC administration (+1.0 THC). The p value for the main effect of THC treatment is shown in each panel (see below for full 2-way ANOVA results). ANOVA results for each panel were as follows: (A) significant effect of dose [F(3,15)=8.214; p=0.002]; (B) no main effect of THC [F(1,5)=1.796; p=0.238] or day [F(5,25)=0.5944; p=0.704], but there was a significant interaction [F(5,25)=5.163; p=0.002]; (C) significant effect of dose [F(3,15)=20.16; p<0.001]; (D) significant effect of dose [F(3,15)=29.30; p<0.001], (E) no main effect of THC [F(1,5)=4.973; p=0.077] or day [F(5,25)=0.229; p=0.946], and no significant interaction

[F(5,25)=1.498; p=0.226]; (F) significant effect of dose [F(3,15)=6.569; p=0.005]. All points show mean ± SEM from 6 rats.

IV.6 Effects of gabapentin on ICSS 7-14 days after bilateral intraplantar saline (A-C) or 5% formalin (D-F). Panels A, C, D and F show effects of cumulative gabapentin (3.2-32 mg/kg) administered on day 7 (A,D) or day 14 (C,F) after intraplantar treatment. Abscissae: dose of gabapentin in mg/kg. Baseline (BL) ICSS determined before gabapentin treatment is also shown in each panel. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. Asterisks indicate significantly different from the daily baseline as determined by a significant one-way ANOVA followed by the Dunnett post hoc test ($p<0.05$; see ANOVA results below). Panels B and E show effects of 32 mg/kg gabapentin administered on days 8-13 after intraplantar treatment. Abscissae: day after intraplantar treatment. Ordinates: total number of stimulations per component, expressed as a percentage of pretreatment baseline. For each day, data are shown for ICSS before morphine administration (Daily Baseline) and after gabapentin administration (+32 gabapentin). The p value for the main effect of gabapetin treatment is shown in each panel (see below for full 2-way ANOVA results). ANOVA results for each panel were as follows: (A) significant effect of dose [F(3,15)=5.289; p=0.011]; (B) a significant main effect of gabapentin [F(1,5)=10.16; p=0.024] but not day [F(5,25)=0.3089; p=0.903)], and no interaction [F(5,25)=0.08467; p=0.994]; (C) significant effect of dose [F(3,15)=20.13; p<0.001]; (D) significant effect of dose [F(3,15)=8.654; p= 0.001], (E) significant main effect of gabapentin [F(1,5)=16.46; p=0.001] but not of day [F(5,25)=1.214; p=0.332], and no interaction [F5,25)=0.5279; p=0.753]; (F) significant effect of dose [F(3,15)=30.78; p<0.001]. All points show mean ± SEM from 6 rats.

IV.7 Effects of morphine, ketoprofen, bupropion, THC, and gabapentin on formalin-induced mechanical hypersensitivity (or allodynia) 7 days after formalin-treatment. Panel A: Magnitude of formalin-induced decrease in paw-withdrawal thresholds for all rats in the study. Abscissa: Pre- or 7-day Post-formalin treatment conditions. Ordinate: paw withdrawal threshold from von Frey filaments in grams (log scale). Formalin significantly decreased thresholds ($t=13.83$, $df=35$; $p<0.001$). All bars show mean ± SEM for 24 rats. Panel B: Effects of test drugs. Abscissa: dose of drug in mg/kg (log scale). Ordinate: paw withdrawal threshold from von Frey filaments in grams (log scale). Filled symbols indicate significantly different from the vehicle as determined by a significant one-way ANOVA followed by the Dunnett post hoc test ($p<0.05$). ANOVA results were as follows: morphine: [$F(5,25)=6.835$; $p=0.004$]; ketoprofen [$F(5,25)=0.270$; $p=0.846$]; bupropion [$F(5,25)=40.133$; $p<0.001$]; THC [$F(5,25)=7.932$; $p=0.003$]; THC: a significant main effect of dose [$F(5,25)=7.932$; $p=0.003$]; gabapentin [$F(5,25)=11.392$; $p<0.001$]. All points show mean ± SEM from 6 rats.

Figure IV.1

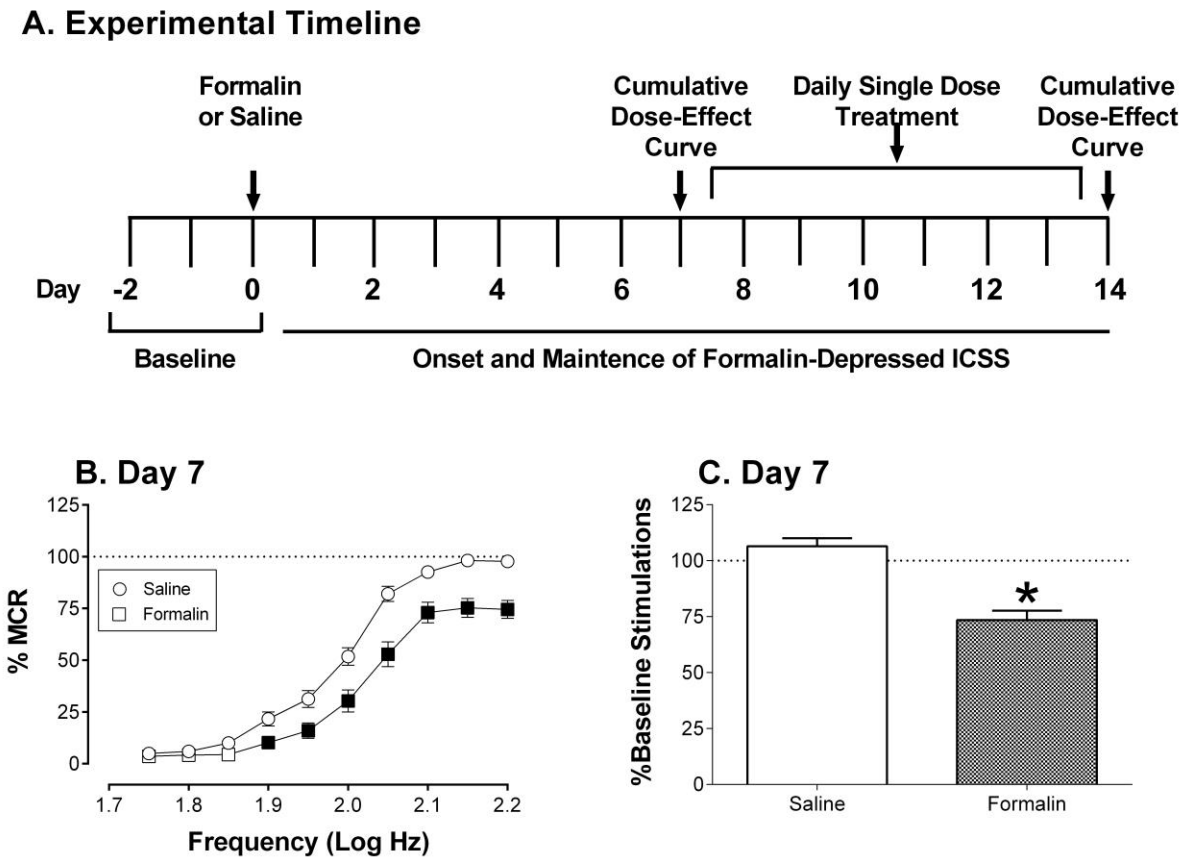

A. Experimental Timeline
Formalin or Saline
Cumulative Dose-Effect Curve
Daily Single Dose Treatment
Cumulative Dose-Effect Curve
Day
-2
0
2
4
6
8
10
12
14
Baseline
Onset and Maintence of Formalin-Depressed ICSS
B. Day 7
% MCR
Saline
Formalin
Frequency (Log Hz)
C. Day 7
%Baseline Stimulations
Saline
Formalin
*

Figure IV.2: Morphine

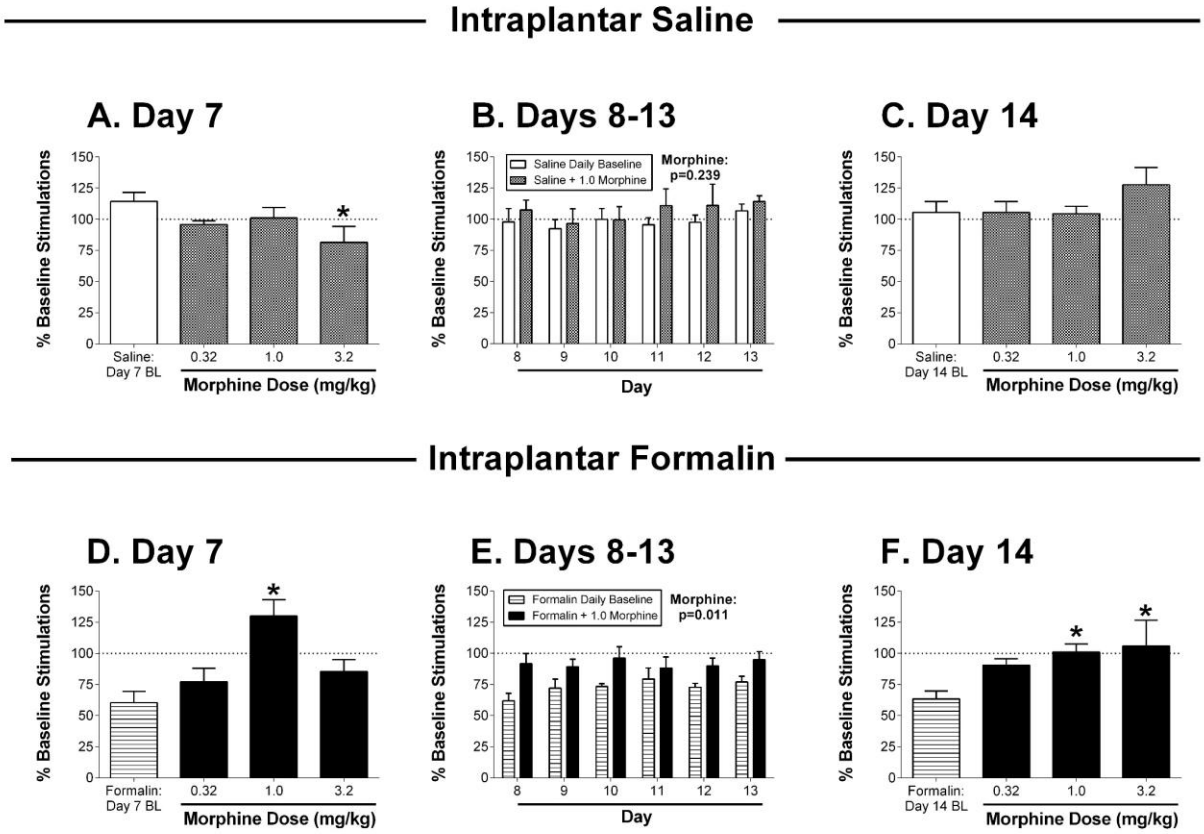


Figure IV.3: Ketoprofen

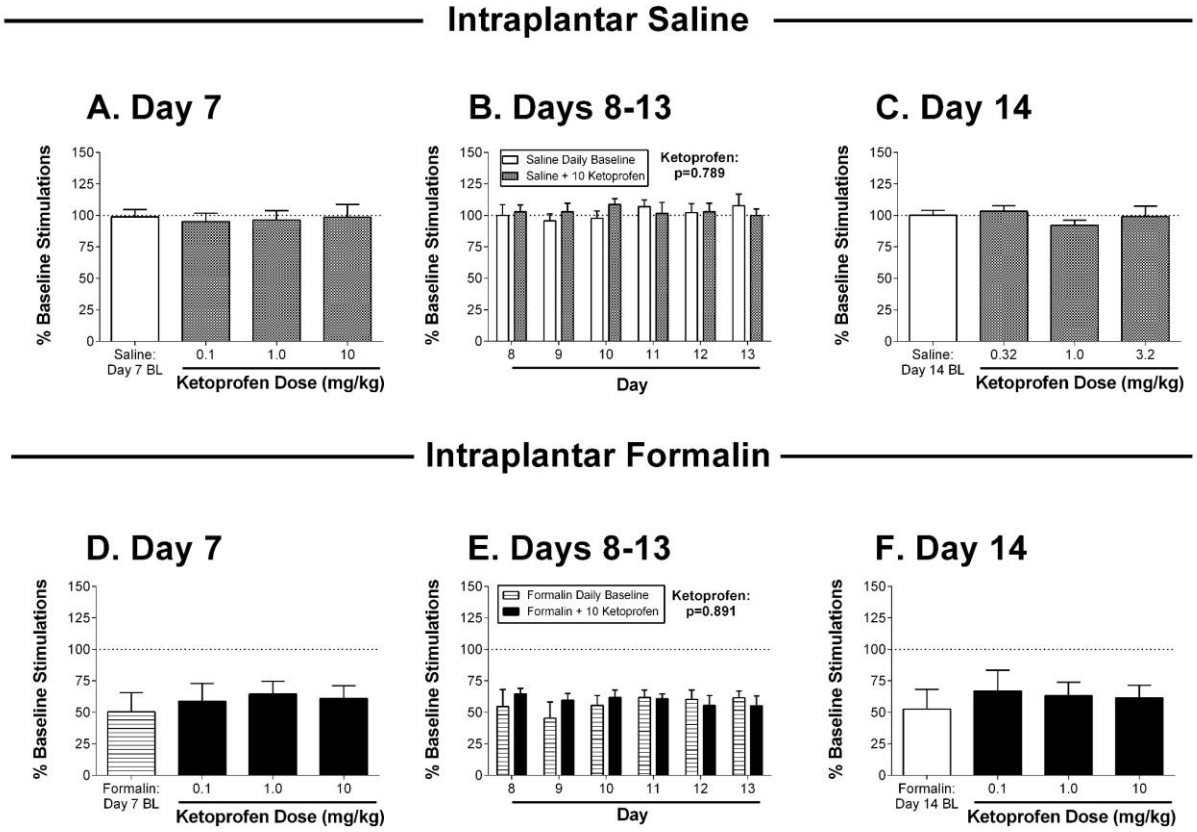


Figure IV.4: Bupropion

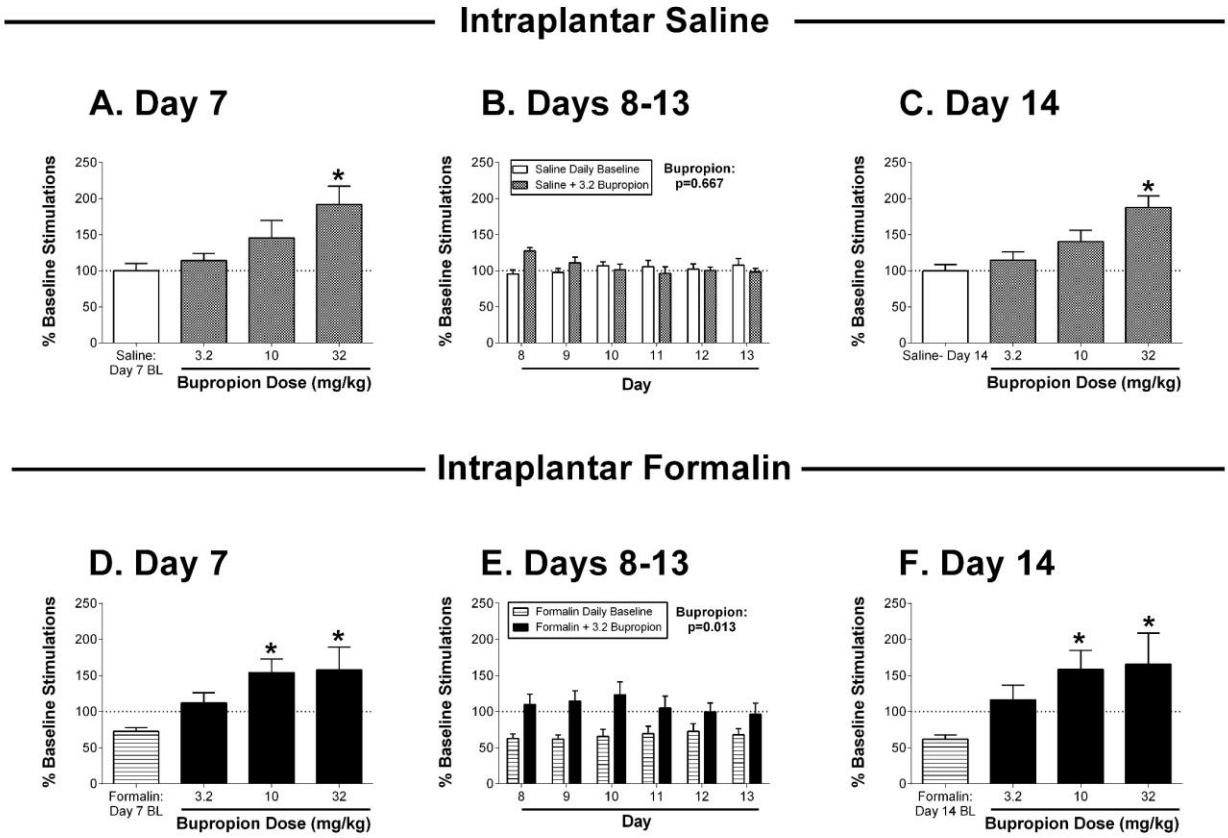


Figure IV.5: THC

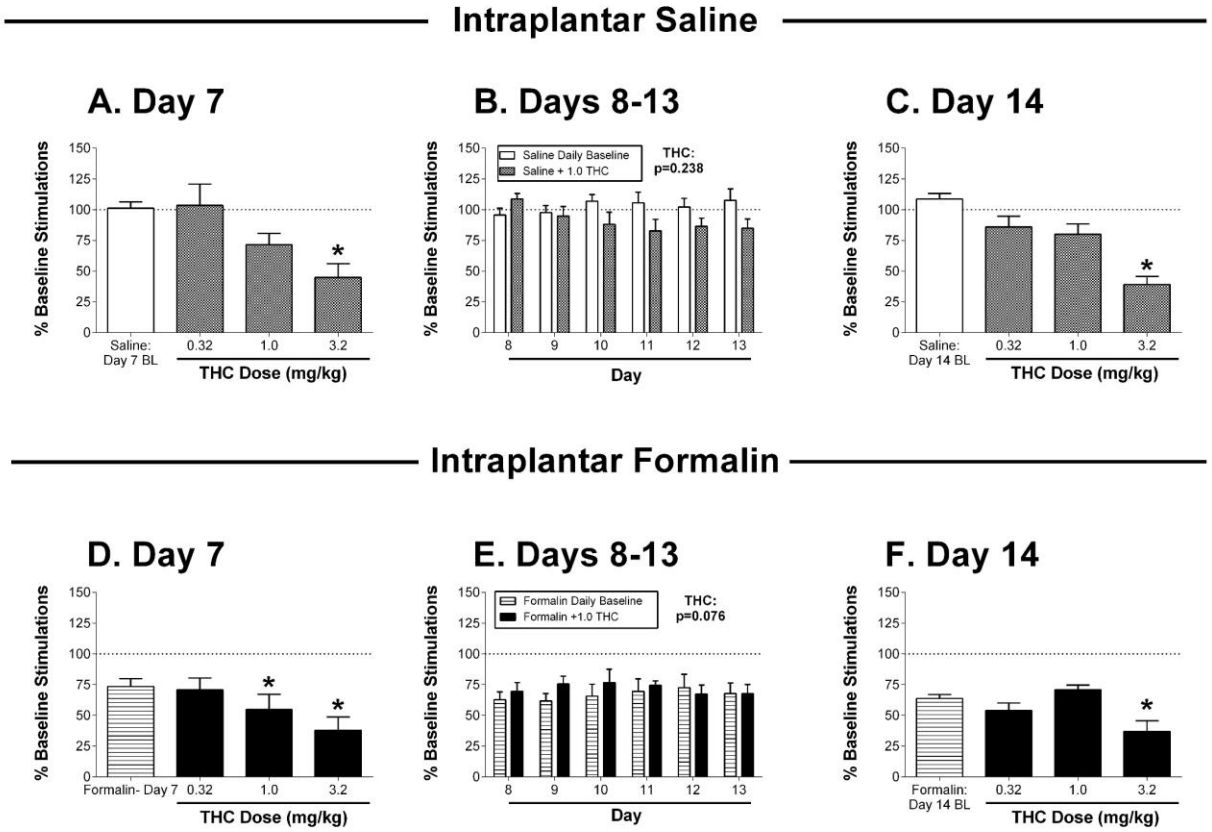


Figure IV.6: Gabapentin

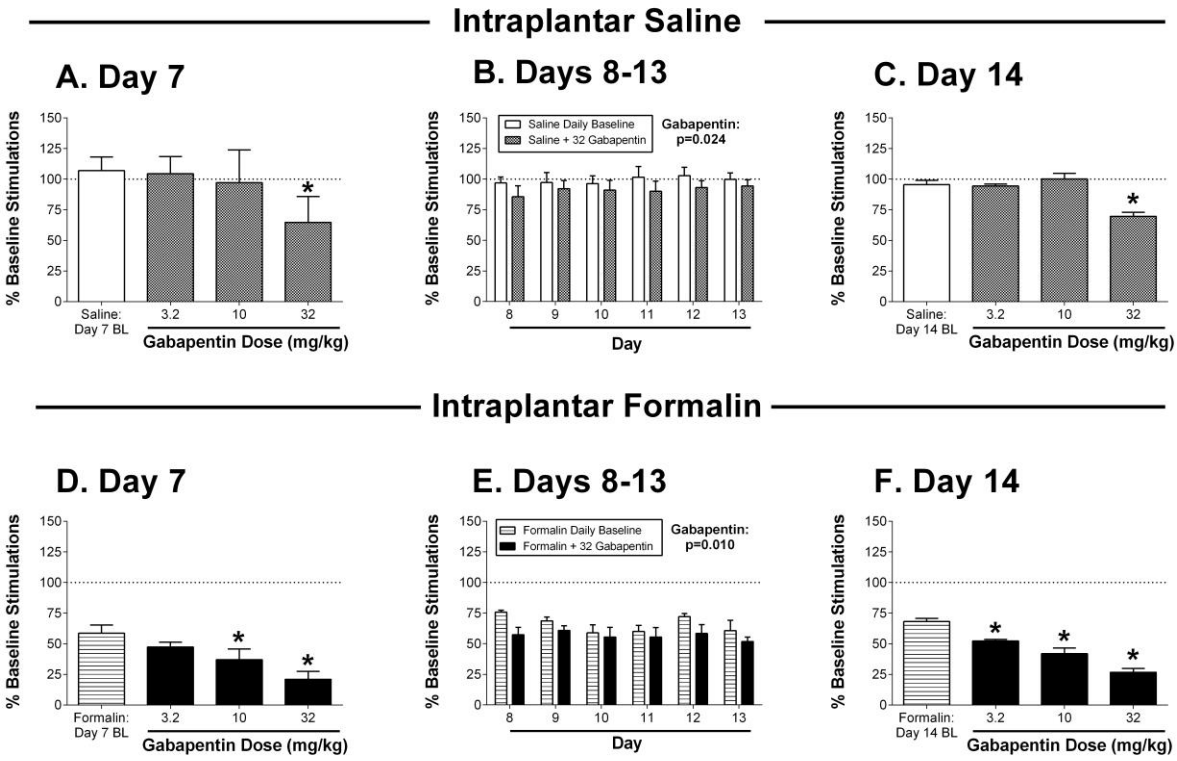
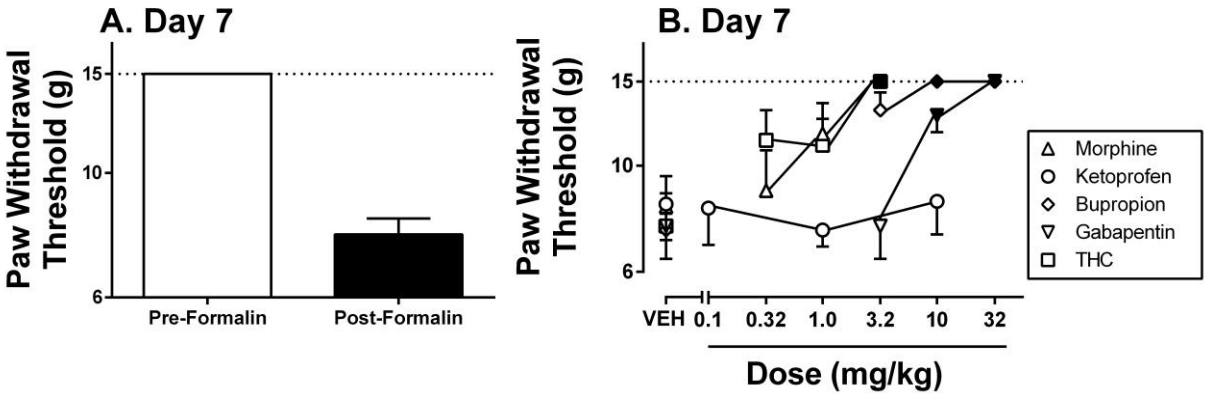


Figure IV.7: Mechanical Allodynia



Chapter V: Discussion

Pain-related depression of behavior. Chapter II studies were conducted to test the hypothesis that depression of behavior in rats caused by IP acid as an acute visceral noxious stimulus is mediated by depression of mesolimbic dopamine release in nucleus accumbens. In agreement with previous studies (Kwilasz *et al*, 2012; Negus *et al*, 2010b; Negus *et al*, 2012; Pereira Do Carmo *et al*, 2009), data presented in Chapter II showed that IP administration of dilute lactic acid served as a noxious stimulus to produce an analgesic-reversible depression of ICSS. This study extended previous findings by using a higher intensity noxious stimulus (5.6% vs. 1.8% lactic acid, see below), and despite use of this higher intensity stimulus, both ketoprofen and morphine retained efficacy to block acid-induced depression of ICSS. These results also agree with previous studies showing pain-related and analgesic-reversible depression of other behaviors including feeding (Kwilasz *et al*, 2012; Stevenson *et al*, 2006), locomotion (Cobos *et al*, 2012; Stevenson *et al*, 2009), burrowing (Andrews *et al*, 2012), and positively reinforced operant responding (Martin *et al*, 2004).

Pain-related depression of mesolimbic DA release. As shown in Chapter II, acid-induced depression of ICSS was accompanied by acid-induced depression of NAc DA levels. In this regard, effects of the acid noxious stimulus were similar to effects of the kappa agonist U69593, and as will be discussed further below, that similarity provided one rationale for the hypothesis that acid effects were mediated by activation of the endogenous dynorphin/KOR system. Before addressing that issue, though, it is relevant first to consider the relationship between the time course and potency of neurochemical and behavioral effects produced by IP acid and U69593. First, regarding time course, both acid and U69593 produced an initial period of declining DA levels followed by a later period of relatively sustained but reduced DA levels.

The time course of ICSS depression corresponded more closely to the time course of declining rather than absolute DA levels, although significant depression of behavior was observed before significant depression of NAc DA levels. A similar temporal relationship has been reported previously for the neurochemical and behavioral effects of the kappa agonist salvinorin A (Carlezon *et al*, 2006; Zhang *et al*, 2005). Second, regarding potency, IP acid was more potent to depress ICSS than NAc DA levels. For example, we have reported previously that a concentration of 1.8% lactic acid was sufficient to significantly depress ICSS (Pereira Do Carmo *et al*, 2009). Although this concentration of acid reduced mean NAc DA levels in preliminary experiments for this study, the reduction was not statistically significant (mean±SEM % baseline DA=95.64±3.87 after saline and 88.15±3.8 after 1.8% acid; p=0.21 by t-test), so a higher intensity stimulus of 5.6% acid was tested that did significantly decrease both ICSS and NAc DA. Kappa agonists also tend to be less potent to depress microdialysis measures of NAc DA than ICSS and other behavioral endpoints (Carlezon *et al*, 2006; Negus *et al*, 2010b; Zhang *et al*, 2005). Moreover, the slower onset, longer duration and lower potency of these treatments to depress NAc DA levels vs. behavior is mirrored by a similar slower onset, longer duration and lower potency of amphetamine-like drugs to stimulate NAc DA vs. behavior (e.g. amphetamine, see (Bauer *et al*, 2013; Schad *et al*, 1995)). Taken together, these results suggest that microdialysis measures of NAc DA are slightly less sensitive to, and slower to recover from, experimental manipulations than ICSS or other behavioral measures. This difference may involve the lag between treatment-induced changes in synaptic DA (which affect behavior) and detection by microdialysis of later changes in extra-synaptic DA levels.

This is the first study to report a pain-related and analgesic-reversible decrease in microdialysis measures of NAc DA after treatment with a noxious stimulus. However, two other

recent studies reported increases in NAc DA release after antinociceptive treatments in rat models of post-surgical or cephalic pain (De Felice *et al*, 2013; Navratilova *et al*, 2013). This preclinical evidence for reciprocal effects of pain- and analgesia-related manipulations on NAc DA corresponds to clinical evidence from functional magnetic resonance imaging studies for reciprocal negative/positive signals in NAc at pain onset/offset, respectively (Becerra and Borsook, 2008). Moreover, these findings agree with other clinical evidence for a negative correlation between pain and mesolimbic DA in humans (Borsook *et al*, 2007; Jarcho *et al*, 2012; Wood, 2008).

In the present study, ketoprofen completely blocked acid-induced depression of both NAc DA and ICSS at a dose that did not alter either NAc DA or ICSS when ketoprofen was administered alone. This finding agrees with previous reports that ketoprofen blocks acid-induced depression of ICSS and supports the proposition that ketoprofen reduced sensitivity to the acid noxious stimulus (Negus *et al*, 2010b; Kwilasz and Negus, 2012). Morphine also blocked acid-induced depression of NAc DA and ICSS at a dose that had no significant effect on NAc DA or ICSS; however, mean DA levels were increased by morphine in this study, and previous microdialysis studies have reported that similar morphine doses produced significant increases in NAc DA (e.g. Cadoni C and DiChiara G, 2007). In addition, 3.2 mg/kg morphine can produce significant facilitation of ICSS depending on variables such as pretreatment time and history of opioid exposure (Altarifi and Negus, 2011). Accordingly, morphine blockade of acid effects on NAc DA and ICSS may reflect both reduced sensitivity to the noxious stimulus and additivity of opposing acid and morphine effects on NAc DA and ICSS.

The finding that IP acid depressed NAc DA contrasts with several earlier microdialysis studies in rodents showing stimulation in NAc DA release by aversive stimuli such as foot shock,

tail shock and tail pinch (Amato *et al*, 2011; Kalivas and Duffy, 1995; Marinelli *et al*, 2005). Resolution of this discrepancy will require further research, but two issues will be highlighted here as potentially important factors. First, aversive stimuli that stimulated NAc DA have all been applied cutaneously, whereas the present study used a visceral noxious stimulus. Cutaneous stimuli originating outside the body are more easily escaped than visceral stimuli and might therefore be likely to stimulate rather than depress behavior and neurochemical systems such as the mesolimbic DA system that mediate behavioral activation. For example, 20-min exposure to tail pinch stimulated both NAc DA levels and locomotor activity in rats (Amato *et al*, 2011). Second, the present study found that IP acid-induced depression of ICSS and NAc DA release was blocked by analgesic drugs, supporting the relationship of these effects to clinical pain. In contrast, analgesics have not been evaluated for their ability to block stimulation of NAc DA release by cutaneous aversive stimuli.

Role of endogenous dynorphin/KOR systems. Results presented in Chapter II with U69593 agree with numerous previous studies in showing that kappa receptor activation is clearly sufficient to depress both ICSS and NAc DA release (Carlezon *et al*, 2006; Negus *et al*, 2010b; Todtenkopf *et al*, 2004; Zhang *et al*, 2005). However, a dose of norBNI that fully blocked these effects of U69593 failed to block IP acid-induced depression of ICSS and NAc DA release. Conversely, U69593 effects were not blocked by a dose of ketoprofen that did block acid effects, and morphine was also more effective to block effects of acid than of U69593. This double dissociation suggests that the endogenous dynorphin/KOR system is not necessary for acid-induced depression of ICSS and NAc DA.

This conclusion is also consistent with the finding that IP acid did not elevate PDYN in NAc or other brain regions at 1.5 or 24 hr after acid administration, and did not alter KOR at any

time. However, the present results do suggest a pain-related perturbation in kappa opioid systems, and behavioral consequences of that perturbation remain to be determined. We focused on adaptations in mesocorticolimbic PDYN and KOR expression because previous work showed that non-noxious stressors activate the transcription factor CREB (cAMP response element binding protein) in the NAc, and that CREB-mediated increases in dynorphin function in this region contribute to depressive-like behavioral signs including anhedonia in the ICSS test (Chartoff *et al*, 2009; Muschamp *et al*, 2011; Pliakas *et al*, 2001). The acid noxious stimulus did not elevate NAc PDYN expression like the stressors tested in these earlier studies, but it did significantly increase PFC PDYN expression at the delayed time point (4 days). Although this effect was significant despite the use of conservative post hoc tests and alpha levels, we acknowledge that there is a possibility of Type I error whenever large numbers of comparisons are made, and future studies will follow up these early results with a more detailed characterization of the time course of this effect, as well as analysis of other proteins. Nevertheless, these data suggest that a visceral noxious stimulus that depresses ICSS may also trigger delayed but more sustained changes (e.g., those in the PFC) that increase vulnerability to depressive-like behaviors at later timepoints (e.g., days after the initial pain stimulus). For example, recent work shows that KOR activation in the PFC causes local reductions in DA levels and establishes conditioned place aversions (Tejeda *et al*, 2013), suggesting that elevated dynorphin function in this region can produce another hallmark sign of depressive illness (dysphoria). These data provide a rationale for future work in which vulnerability to depressive behavior is studied at time points far beyond the acute effects of a painful/stressful stimulus (Knoll *et al*, 2010). Additionally, the fact that our data demonstrate that acute pain can cause adaptations within the mesocorticolimbic system opens the door to the study of other target

genes that are implicated in depressive behavior but that would be expected to be minimally sensitive to KOR blockade [e.g., BDNF; see (Berton *et al*, 2006)].

Expression of chronic pain-depressed behavior and the role of endogenous kappa opioid system activation in rats.

Chapter II evaluated expression and mechanisms of behavioral depression produced by an acute pain stimulus. However, clinically relevant depression of behavior by pain is usually associated with more chronic inflammatory or neuropathic pain states. Accordingly, Chapter III evaluated the hypothesis that inflammatory and neuropathic challenges thought to produce sustained or chronic pain would also produce sustained depression of behavior. In doing so, a comparison of CFA and formalin were made, and a variety of dependent measures (physiological and operant) were evaluated.

CFA-and formalin effects on paw width, mechanical allodynia and body weight.

The CFA and formalin effects reported in Chapter III agree with previous studies in rats that examined the time course of paw swelling and/or mechanical sensitivity after intraplantar CFA (Stein et al., 1988; Chaplan et al., 1994; Grace et al., 2014) or formalin (Fu et al., 2001; Fu et al., 2000; Grace et al., 2014). For example, (Fu et al., 2001) demonstrated that a 5% formalin injection into the hindpaw of rats produced both mechanical and thermal allodynia for up to four weeks following administration. Similarly, (Grace et al., 2014) found that bilateral injection of either CFA or formalin into the hindpaw resulted in mechanical allodynia that lasted up to seven days. Transient weight loss in CFA-treated rats, but not formalin-treated rats, is also consistent with previous studies. For example, 100µl CFA administered to the tail-base in rats produced a magnitude and time course of weight loss similar to that reported here (Rofe et al., 1990),

whereas rats gained weight normally for six weeks after unilateral intraplantar injection of 50 µl 5% formalin (Vierck et al., 2008).

Differential effects of CFA and formalin on ICSS. Although CFA and formalin produced similar effects on mechanical allodynia as a measure of pain-stimulated behavior, they produced distinct effects on depression of ICSS as a measure of pain-depressed behavior. The greater and more sustained efficacy of formalin to depress ICSS may be related to its induction of necrosis in the paw, neuropathy of primary afferents, and/or microglial activation at the level of the spinal cord (Winter and McCarson, 2005; Lin et al., 2007; Berta et al., 2014), and we are actively investigating the role of these formalin effects in formalin-induced depression of ICSS. However, regardless of mechanism, these results extend the range of pain-related stimuli that have been found to depress brain reward function as assessed with ICSS in rats, and further identify bilateral intraplantar formalin as the stimulus producing the most sustained depression of ICSS so far reported. For example, previous studies have shown transient (1-2 hr) pain-related and analgesic-reversible depression of ICSS by IP injection of dilute acid (Do Carmo et al., 2009; Negus, 2013), and ICSS was also depressed for up to three hours by intraplantar CFA (present study) and for up to two days by paw incision (Ewan and Martin, 2014). In contrast, effects of bilateral intraplantar formalin in the present study lasted for at least 14 days. Moreover, the poor efficacy of unilateral intraplantar formalin to alter ICSS in this study agrees with the finding that a unilateral spinal nerve ligation-model of neuropathy also failed to alter ICSS at any time (Ewan and Martin, 2014).

The present evaluation of CFA and formalin effects on ICSS also warrant comparison to CFA and formalin effects on some other metrics of pain-related behavioral depression and/or negative affective states. For example, unilateral treatment in rats with intraplantar CFA doses

similar to that used here depressed diurnal exploratory activity for four weeks (Larsen and Arnt, 1985) and burrowing for 10 days (Andrews et al., 2012); however, pain-related changes in facial expression or place conditioning were apparent for only one day (Sotocinal et al., 2011; Okun et al., 2011), and neither nocturnal locomotor activity nor wheel running were significantly affected at any time (Larsen and Arnt, 1985; Grace et al., 2014). Bilateral CFA injection, such as that used in the present study, did depress both nocturnal locomotor activity (for four weeks) and wheel running (for two days) in rats, and studies in mice have also reported a requirement for bilateral CFA treatment to produce transient depression of wheel running (Cobos et al., 2012). Taken together, these results indicate that CFA has different efficacies and time courses to produce different pain-related behaviors, and ICSS in rats is relatively resistant to CFA effects.

Fewer studies have examined effects of formalin in procedures of pain-related behavioral depression and/or negative affective states. Perhaps of greatest relevance to the present study, bilateral intraplantar formalin produced avoidance for six weeks of noxious thermal stimuli in an operant-escape procedure (Vierck et al., 2008). Intraplantar formalin has also been shown to produce pain-related changes in facial expression and place conditioning (Langford et al., 2010; Johansen et al., 2001; Xiao et al., 2013), but these effects were evaluated only for the first hour after formalin administration, and more sustained formalin effects on these procedures have not been examined. Lastly, in contrast to formalin effects on ICSS, bilateral intraplantar formalin administration had no effect on wheel running in rats (Grace et al., 2014). This distinction is notable, because the failure of bilateral intraplantar formalin to alter either body weight (present study) or wheel running (Grace et al., 2014) provides evidence to suggest that ICSS depression by formalin could not be attributed to general behavioral impairment.

Morphine reversal of formalin-induced depression of ICSS. The failure of morphine to significantly alter ICSS in rats after intraplantar saline treatment is consistent with previous studies showing little or no effect of these morphine doses on ICSS in opioid-naïve rats (Do Carmo et al., 2009; Negus et al., 2010b; Altarifi et al., 2012). However, these same morphine doses significantly reversed formalin-induced depression of ICSS, consistent with previous studies showing that morphine also blocks acute depression of ICSS by IP acid (Do Carmo et al., 2009; Negus et al., 2010a). Moreover, the high potency of morphine to block formalin-induced depression of ICSS (effective at 0.32 mg/kg) is similar to the high potency of morphine to block acid-induced depression of ICSS (Do Carmo et al., 2009). Reversal of formalin-induced depression of ICSS by the opioid analgesic morphine provides one source of evidence to suggest that this formalin effect may be related to sustained pain.

In the present study, high morphine doses not only reversed formalin-induced depression of ICSS but also increased ICSS above original baseline levels. Mechanisms responsible for this morphine effect are not currently known; however, the emergence of rate-increasing effects produced by these morphine doses after formalin treatment is similar to the emergence or enhancement of rate-increasing effects produced by regimens of prior morphine exposure (Altarifi and Negus, 2011). Formalin treatment has been reported to promote endogenous opioid release (Kuraishi et al., 1984; Bourgoin et al., 1990; Zangen et al., 1998), and this raises the possibility that endogenous opioid release stimulated by formalin treatment had the effect of sensitizing rats to rate-increasing effects of subsequent treatment with the exogenous opioid morphine.

NorBNI failed to reverse formalin-induced depression of ICSS. Administration of the endogenous kappa agonist dynorphin or of exogenous kappa agonists like salvinorin A is

sufficient to decrease mesolimbic DA release and to depress ICSS in rodents (Yokoo et al., 1994; Carlezon, 2005; Todtenkopf et al., 2004; Negus et al., 2010b). In addition, previous studies have shown that some non-pain stressors can increase central biomarkers for kappa opioid function and produce depression-like behaviors that can be blocked by kappa antagonists (Mague et al., 2003; Chartoff et al., 2009; Bruchas et al., 2010; Van't Veer and Carlezon, 2013). These findings have suggested the possibility that activation of endogenous kappa opioid systems might also mediate pain-related depression of ICSS. Accordingly, the present study tested the hypothesis that CFA and/or formalin might activate endogenous kappa opioid signaling and produce kappa antagonist-reversible depression of ICSS. However, the present results do not support this hypothesis for four reasons. First, neither CFA nor formalin significantly increased central PDYN or KOR mRNA levels. Second, although this analysis may have failed to detect small but real changes in kappa biomarkers (a Type II error), there was no pattern for either a trend toward increased biomarker levels or a difference in CFA and formalin effects on biomarkers consistent with the difference in their effects on ICSS. Third, CFA- and formalin-induced changes in PDYN never approached the nearly two-fold increase in PDYN produced in rats exposed to the stress of a forced swim test (Chartoff et al., 2009). Finally, the formalin-induced decrease in ICSS was not blocked by the kappa antagonist norBNI, suggesting that any modest effects that formalin might have had on kappa biomarkers were not sufficient to produce a kappa receptor-mediated decrease in ICSS.

The failure of norBNI, to reverse formalin-induced depression of ICSS suggested that non-kappa mechanisms were responsible for formalin-induced depression of ICSS. In effort to follow-up on these results, we subsequently characterized the pharmacological modulation of formalin-depressed by ICSS by drugs from different pharmacological classes to test possible

alternative mechanisms in Chapter IV. Endpoints in these studies consisted of classically used pain-stimulated (or reflex-withdrawal) dependent measures (e.g. threshold mechanical stimulation with von Frey filaments required to elicit paw withdrawal) in addition to pain-depressed operant responding following acute and repeated administrations of each drug.

Formalin-induced mechanical allodynia and depression of ICSS. The effects of formalin on the production of mechanical allodynia, or hypersensitivity to a normally non-noxious stimulus, were evaluated in this study, and the results obtained were consistent with previous studies employing a formalin dose of a sufficient intensity (Leitl et al., 2014; Fu et al., 2000). Here, a dose of 5% formalin was sufficient to produce a hypersensitivity that remained present for at least 14 days. To further characterize the effects of formalin induced allodynia, we tested a range of prototypical and experimental analgesics. Pain-depressed operant responding of ICSS behavior appears to be sensitive to modulation by some noxious stimuli, but not all purported noxious stimuli or sub-chronic stimuli (Leitl et al., 2014a). In these studies, we used intraplantar formalin to decrease operant response rates; previous studies from our lab have shown formalin produced decreases in behavior that were reversed by doses of the mu opioid analgesic morphine at doses that did not alter control responding (Leitl et al., 2014a).

Morphine effects. Morphine is a clinically effective analgesic and agonist at mu opioid receptors. Morphine dose-dependently reversed both formalin-stimulated mechanical allodynia and formalin-induced depression of ICSS 7 days following formalin administration. This agrees with Chapter III results showing that morphine is capable of acutely reversing formalin-induced depression of ICSS on day 7 (Leitl et al., 2014a), and Chapter IV results extend on this finding by showing that morphine retained its effectiveness to block formalin-induced depression of ICSS during repeated morphine treatment. The effectiveness of the analgesic morphine to block

both formalin-stimulated mechanical allodynia and formalin-induced depression of ICSS is consistent with the interpretation that depression of ICSS by formalin is related to pain. Interestingly, doses of morphine (1.0 mg/kg) that were effective in reversing formalin-induced pain measures did not alter ICSS responding in control rats (intraplantar saline) to an appreciable (or statistically significant) degree. These results suggest formalin-treated rats may be more sensitive to mu opioid analgesic morphine than rats that are not in a purported pain-state. Moreover, the sustained effectiveness of morphine to reverse formalin-induced depression of ICSS during repeated morphine treatment is consistent with other evidence to suggest that morphine antinociception is resistant to tolerance in assays of pain-depressed behavior (Altarifi and Negus, 2015) and may also agree with evidence for sustained analgesic effectiveness of morphine in many clinical contexts (Harden et al., 2010; Morgan and Christie, 2011).

Ketoprofen effects. Ketoprofen is an NSAID, and NSAIDs are a class of analgesics that are defined by an ability to inhibit prostaglandin synthesis by blocking the COX enzymes necessary to produce prostaglandins (McQuay, 2007). NSAIDs, including ketoprofen, have four main pharmacological effects: anti-inflammatory, analgesic, antipyretic, and anti-thrombotic. Ketoprofen has previously been shown to block ICSS depression following acute delivery of IP acid (Leitl et al., 2014b), but was not sufficient to block ICSS depression following delivery of a sustained noxious stimulus. The failure of ketoprofen to block formalin-induced depression of ICSS or mechanical allodynia suggests that formalin is producing sustained depression of ICSS and mechanical allodynia through an inflammation-independent mechanism such as neuropathy; this is further corroborated in by weak efficacy of NSAIDs in the treatment of chronic neuropathic pain states in human clinical studies (De Leon-Casasola, 2013; Fornasari, 2012). In

sum, neuropathy, but not inflammation, appears to be the driving force behind formalin-induced depression of ICSS.

Bupropion effects. Bupropion is a DA/NE uptake inhibitor used clinically as an antidepressant (Semenchuk et al., 2001). Bupropion has also been shown to block acid-induced depression of ICSS (Rosenberg et al., 2013). In Chapter II, we showed induction of a pain-state resulted in a hypodopaminergic state in the NAc, and it has previously been shown that bupropion increases DA levels in the NAc (Sidhpura et al., 2007). The effectiveness of bupropion to also reverse formalin-induced depression of ICSS suggests that sustained formalin-induced depression of ICSS may also involve a hypodopaminergic state. Moreover, as with morphine, bupropion retained effectiveness during repeated administration, suggesting that tolerance does not develop to the antinociceptive effects of bupropion in this procedure. Bupropion was also able to dose-dependently reverse mechanical allodynia in addition to dose-dependently reversing formalin-induced depression of ICSS 7 days following formalin administration. This study expands upon previous study showing that bupropion blocks a pain-stimulated behavior (i.e. stretching) elicited by an acute, visceral noxious stimulus (lactic acid) (Rosenberg et al., 2013). These results are also in agreement with evidence demonstrating bupropion (and some other monoamine uptake inhibitors) have clinical efficacy to treat neuropathic pain (Finnerup et al., 2015; Semenchuk et al., 2001). Interestingly doses of bupropion (3.2-10 mg/kg) that were effective in reversing formalin-induced pain measures did not alter ICSS responding in control rats (intraplantar saline) to an appreciable (or statistically significant) degree. These results suggest formalin-treated rats are more sensitive to the analgesic effects of the DA/NE inhibitor bupropion than rats that are not in a purported pain-state.

THC effects. THC and other natural cannabinoids stem from the marijuana plant (*Cannabis sativa*), and THC itself is an agonist at cannabinoid 1 and cannabinoid 2 receptors (Axelrod and Felder, 1998). THC and other cannabinoid receptor agonists have been studied extensively with the intent of characterizing their potential therapeutic properties. Although the marijuana plant itself is widely used by humans, and although THC and other cannabinoids often appear analgesic in preclinical studies, there is poor evidence supporting its use in the clinic due to poor efficacy and high incidence of adverse effects (Beaulieu & Ware, 2007; Finnerup et al., 2015; FASAM et al., 2005). In the studies conducted for this dissertation, THC was able to dose-dependently reverse mechanical allodynia, a finding that agrees with previous studies that evaluated effects of THC on mechanical allodynia elicited by neuropathic manipulations (Brownjohn and Ashton, 2012)(Craft et al., 2013). However, THC doses that blocked mechanical allodynia also decreased control (intraplantar saline) ICSS. Additionally, THC (1.0-3.2 mg/kg) exacerbated formalin-induced depression of ICSS on day 7. Following repeated treatment of THC (1.0 mg/kg on Day 8-13), re-determination of the dose-response function did not reveal tolerance to the rate decreasing effects of THC on formalin-depressed ICSS, and formalin-induced depression of ICSS was exacerbated again on day 14, albeit at a slightly higher dose (3.2 mg/kg) than day 7. The apparent efficacy of THC on mechanical allodynia should be viewed with caution and in parallel with its inability to reverse pain-related depression of a positively reinforced operant procedure. In particular, the similar potencies of THC to reduce mechanical allodynia, reduce control ICSS, and exacerbate formalin-induced depression of ICSS suggests that THC effects on mechanical allodynia reflect motor impairment rather than analgesia. Furthermore, these results are in agreement with clinical data that suggests THC is generally not recommend as a first-line therapy in human patients based on a high number

needed to treat relative to the number of adverse events that are observed clinically (Finnerup et al., 2015).

Gabapentin effects. Gabapentin is used clinically as an anticonvulsant, and although its precise mechanism(s) of action remain a topic of research, it is generally thought binding to the alpha(2)delta subunit of voltage-gated calcium channels contributes to antinociceptive properties (Urban, 2005). Similar to THC, gabapentin was able to dose-dependently reverse mechanical allodynia while decreasing control (intraplantar saline) ICSS. Additionally, gabapentin exacerbated, or further reduced formalin-induced depression of ICSS on day 6. Following repeated treatment of gabapentin (32 mg/kg on Day 8-13), and re-determination of the dose-response function, it did not reveal tolerance to the rate decreasing effects of gabapentin on formalin-depressed ICSS. The apparent efficacy of gabapentin to alleviate mechanical allodynia should also be viewed with caution and in parallel with its inability to reverse formalin-induced depression of ICSS. These results also offer a counterpoint to clinical use of gabapentin for pain treatment. Although gabapentin is commonly recommended as a first-line therapy in humans suffering from chronic and/or neuropathic pain, it shows efficacy in only a small subset of patients, but is reasonably safe and tolerable, thus recommended prior to opiates despite inferior clinical efficacy to opioids and other drugs (Finnerup et al., 2015; Chang et al., 2014).

Conclusions

Pain-related depression of ICSS. In the clinical setting, functional impairment and behavioral depression are common manifestations of pain, and efforts have been made to not only capture these behaviors but also monitor their responsivity to pharmacological modulation

(Cleeland and Ryan, 1994; Melzack, 1975; Turk et al., 2003; Melzack, 1987). Procedures used in this dissertation extend evaluation of pain-related functional impairment and behavioral depression from clinical to preclinical studies. We have evaluated effects of numerous putative pain manipulations on ICSS in rats. ICSS was most reliably depressed by IP lactic acid as an acute and transient inflammatory stimulus and by intraplantar formalin as a more sustained neuropathic stimulus. ICSS was also transiently decreased by intraplantar CFA, and by a paw-incision model of post-surgical pain (Ewan and Martin, 2014). In general, though, pain-related depression of ICSS was weaker and more transient with these manipulations than pain-related stimulation of other behaviors such as mechanical allodynia. Moreover, other putative pain models that produce signs of pain-stimulated behavior have failed to alter ICSS. For example, spinal nerve ligation is a surgical method for modeling neuropathy, and it produced mechanical allodynia but failed to depress ICSS (Ewan and Martin, 2011; Ewan and Martin, 2014), and preliminary studies for this dissertation found that intra-articular administration of CFA into the knee joint (a model for arthritis pain) also produced mechanical allodynia without depressing ICSS. Finally, studies using other behaviors such as wheel-running in mice or rats have also found that pain-related depression of behavior is less sensitive than pain-stimulated behaviors (e.g. mechanical allodynia) to inflammatory or neuropathic manipulations (Grace et al., 2014; Cobos et al., 2012). Taken together, these results suggest that commonly used preclinical pain manipulations often produce weaker and/or more transient signs of pain-depressed behavior than pain-stimulated behavior.

Role of decreased DA signaling in pain-related depression of ICSS. Microdialysis studies performed in this series of research experiments support a relationship between pain-related depression of behavior and pain-related depression of DA after IP lactic acid (Leitl et al.,

2014b). We did not investigate DA levels after intraplantar formalin, but effectiveness of the DA/NE uptake inhibitor bupropion to alleviate both acute IP lactic acid-induced depression of ICSS and sustained intraplantar formalin-induced depression of ICSS suggests that both acute and chronic pain-related depression of ICSS may involve a hypodopaminergic state, in agreement with other evidence for a relationship between pain and reduced mesolimbic DA signaling (Coffeen et al., 2010; Taylor et al., 2015).

Role of dynorphin and KORs as mechanism for pain-related depression of DA. Mesolimbic DA neurons express kappa receptors, and activation of those receptors either by endogenous dynorphin or by exogenous kappa agonists like U69593 can depress both ICSS and mesolimbic DA release (Leitl et al., 2014b; Carlezon et al., 2006). Moreover, previous studies have suggested that activation of this endogenous dynorphin/kappa receptor system by some non-pain stressors can produce signs of behavioral depression (Chartoff et al., 2009; Borsook et al., 2007; Knoll and Carlezon, 2010). However, data reported in this dissertation do not support a role for the dynorphin/kappa receptor system in acute or chronic pain-related depression of ICSS; furthermore no purported pain stimuli examined in these studies reliably altered biomarkers for dynorphin or kappa receptors, and the kappa antagonist was ineffective to block pain-related depression of DA release or ICSS (Leitl et al., 2014b; Leitl et al., 2014a).

Predictive validity of preclinical models of pain-depressed ICSS. In general, these studies demonstrate good concordance with clinical data for NSAIDs, which are effective for inflammatory but not neuropathic pain, and for opioids and monoamine uptake inhibitors with a DA-ergic component, which are effective for both inflammatory and neuropathic pain (Leitl et al., 2014b; Miller et al., 2015; Leitl et al., 2014a; Finnerup et al., 2015; McQuay, 2007; Sarzi-Puttini et al., 2010). However, these procedures were not sensitive to THC and gabapentin, and

this preclinical result is consistent with clinical evidence that these drugs are not effective for the treatment of inflammatory pain and have weak if any effectiveness to treat neuropathic pain (Beaulieu and Ware, 2007; Lynch and Campbell, 2011; Ware et al., 2010; Chang et al., 2014; Finnerup et al., 2015).

Future directions

Studies conducted here support a role for decreased mesolimbic DA signaling in pain-related depression of behavior; however, the mechanisms that mediate decreases in DA signaling by noxious stimuli remain to be determined. Although activation of endogenous kappa opioid systems have emerged as one mechanism whereby some stressors can reduce DA signaling and produce behavioral depression, evidence collected for this dissertation do not support a role for kappa mechanisms in mediating pain-related behavioral depression. Accordingly, it will be necessary to search for other possible mechanisms. One possibility is that formalin-induced depression of ICSS is mediated through glial activation following induction of a neuropathic pain state. Toll-like receptors (TLRs) are commonly known for their expression on immune cells, and for their role in initiating immune responses in the presence of pathogens (Watkins et al., 2009). Increasing evidence suggests glial cells and/or macrophages, as well as primary sensory neurons, are involved in pain sensation. Moreover, formalin-induced neuropathy has been shown to result in an increase in glial activation in the spinal cord. This glial activation and presence of macrophages in response to nerve injury may be partially or fully responsible for sustained pain-related depression of behavior and mechanical hypersensitivity (Ji et al., 2013). Glial inhibitors, including minocycline and ibudalast (AV411) exist, are currently being evaluated, and have shown promising results for the potential treatment of neuropathic pain

(Ellis et al., 2014; Berta et al., 2014; Ledeboer et al., 2006). Studies are underway to evaluate effectiveness of ibudilast in rats to block both formalin-induced glial activation in spinal cord and formalin-induced depression of ICSS.

Chapter VI: References

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