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EFFECTS OF MU OPIOID RECEPTOR AGONISTS ON INTRACRANIAL SELF-STIMULATION IN THE ABSENCE AND PRESENCE OF “PAIN” IN RATS

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

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{وَقُلْ اَعْمَلُوا فَسَيَرَى اللّٰهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ وَسَتُرَدُّونَ اِلَىٰ عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُمْ بِمَا كُنْتُمْ تَعْمَلُونَ}

التوبة 105

{And say, "Do [as you will], for Allah will see your deeds, and [so, will] His Messenger and the believers. And you will be returned to the Knower of the unseen and the witnessed, and He will inform you of what you used to do"} Quran (9,105)

The first and foremost, I want to thank God for giving me the will, health, and desire to pursue his signs and secrets in this world. Being in the field of science makes me closer to him more than ever been.

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LIST OF ABBREVIATIONS

ACC	Anterior Cingulate Cortex
Amg	Amygdala
BPI	Brief Pain Inventory
cAMP	cyclic-Adenosine Monophosphate
CPP	Conditioned Place Preference
DLPT	Dorsolateral Pontine Tegmentum
GABA	gamma-Aminobutyric Acid
GDP	Guanosine Diphosphate
GIRKs	G-Protein Coupled Inwardly-Rectifying Potassium Channels
GTP	Guanosine Triphosphate
GPCR	G-Protein Coupled Receptor
ICSS	Intracranial Self-Stimulation
IP	Intraperitoneal
LA	Lactic Acid
MCR	Maximum Control Rate
NA	Nucleus Accumbens
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NLTX; NTX	Naltrexone
PAG	Periaquiductal Gray
PBN	Parabrachial Nucleus
PN	Primary Nociceptors
RMTg	Rostromedial Tegmentum
RVM	Rostroventral Medulla
SC	Subcutaneous
SAMHSA	Substance Abuse Mental and Health Services Administration
SEM	Standard Error of Mean
TRPV1	Transient Receptor Potential Vanilloid 1
Veh	Vehicle
VTA	Ventral Tegmental Area

Abstract

EFFECTS OF MU OPIOID RECEPTOR AGONISTS ON INTRACRANIAL SELF-STIMULATION IN THE ABSENCE AND PRESENCE OF PAIN IN RATS

By: Ahmad A. Altarifi, Ph.D.

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

Virginia Commonwealth University, 2013

Director: S. Stevens Negus, Ph.D.
Professor, Department of Pharmacology and Toxicology

Pain is a significant health problem. Mu opioid receptor agonists are used clinically as analgesics, but their use is constrained by high abuse liability. Intracranial self-stimulation (ICSS) is a preclinical behavioral procedure that has been used to assess abuse potential of opioids, and drug-induced facilitation of ICSS is interpreted as an abuse-related effect. ICSS can also be used as a behavioral baseline to detect affective dimensions of pain. Specifically, pain-related depression of ICSS can model pain-related depression of behavior and mood, and drug-induced blockade of pain-related ICSS depression can serve as a measure of affective analgesia. This dissertation used mu agonists that vary in efficacy at the mu receptor (methadone> fentanyl> morphine> hydrocodone> buprenorphine> nalbuphine) and compared their effects on ICSS in the absence (phase one) or presence (phase 2) of pain. Adult male Sprague-Dawley rats were equipped with intracranial electrodes targeting the medial forebrain bundle and trained to lever press for brain stimulation. Different frequencies of stimulation maintained a frequency-dependent increase in ICSS rates, and permitted detection of both rate-

increasing and rate-decreasing treatment effects. During phase 1, medium- and high-efficacy mu agonists produced initial rate-decreasing effects, followed by abuse-related rate-increasing effects at later time points. Repeated morphine administration produced tolerance to its own rate-decreasing effects, cross-tolerance to rate-decreasing effects of other mu agonists, and enhanced expression of rate-increasing effects. Low efficacy mu agonists only produced rate-increasing effects, which were enhanced after repeated morphine. These results suggest that previous opioid exposure increases expression of abuse-related facilitation of ICSS by mu agonists regardless of efficacy. During phase 2, intraperitoneal administration of lactic acid (1.8%) served as a noxious stimulus to depress ICSS. All mu agonists blocked acid-induced depression of ICSS at doses similar to those that facilitated ICSS in the absence of pain. A higher intensity noxious stimulus (5.6 % acid) produced further depression of ICSS and reduced the antinociceptive potency of both methadone and nalbuphine. Morphine antinociception was resistant to tolerance in the assay of acid-depressed ICSS. Overall, these results provide a basis for comparing determinants of abuse-related opioid effects in the absence of pain with their affective analgesic effects in the presence of pain.

CHAPTER ONE

Introduction and Background

1.1. Opioid background

Opioids are a group of drugs that are widely used in clinical and veterinary medicine for multiple purposes. The prototype opioid morphine is a naturally occurring alkaloid that can be extracted from the juice that leaks from the poppy plant, *Papaver somniferum*. Many synthetic and semi-synthetic analogues have been synthesized in the last century. The origin of human use of opioids is not exactly known, but there is some evidence that opioids were used by the Greeks, Indians, and Sumerians (Brownstein, 1993). They extracted the juice (called opium) from the poppy seed pods and used it as a euphoriant in religious rituals, as a poison, and medicinally to treat pain and other illnesses (Trescot *et al.*, 2008). In 1804, a German scientist isolated the main active ingredient in opium: morphine (Klockgether-Radke, 2002). The name is derived from Morpheus, after the Greek god of dreams. Later, other naturally occurring opioids were identified, and pharmaceutical companies initiated their sales of morphine. The high abuse potential of morphine encouraged researchers to synthesize compounds that resemble morphine in its beneficial properties, but with lower abuse potential. These compounds have been classified as “semi-synthetic” (i.e. derived from morphine

or other alkaloids found in opium) or “synthetic” (structurally distinct from morphine). Heroin (first synthesized in 1874) was the first semi-synthetic opioid, and despite its popularity at the beginning as a substitute to morphine, it had more potential of being abused and misused by patients. This led to an increase in research focusing on synthesizing alternative compounds to morphine that might produce fewer side effects but maintaining analgesic properties. Table 1.1 summarizes the opioid ligands that were used in this project.

Table 1.1. Classification and date of discovery for mu opioid agonists that are used in this project

Ligand	Classification	Date of discovery
Methadone	Synthetic	1937
Fentanyl	Synthetic	1960
Morphine	Natural	1804
Hydrocodone	Semi-synthetic	1920
Buprenorphine	Semi-synthetic	1980s
Nalbuphine	Semi-synthetic	1960s
Naltrexone	Semi-synthetic	1963

Currently, opioids are among the most effective and reliable tools available to clinicians for the treatment of strong pain associated with acute trauma (e.g. surgery, burns) or chronic pain associated with cancer and other illnesses (Gutstein and Akil, 2005). For example, recent surveys indicate that opioid analgesics account for more than 10% of all prescription drug sales in the U.S. (Max, 2003), and four of the 10 most prescribed drugs in 2005 were opioid analgesics

(<http://www.rxlist.com/script/main/art.asp?articlekey=79509>). In 2009, Vicodin (trade name for hydrocodone containing acetaminophen) was the most prescribed drug in the U.S. (IMS National Prescription Audittm). These statistics show the impact of pain on society, and the important role that opioids can play in this clinically relevant topic.

Despite their clinical importance and significant role in decreasing the “feel of pain”, physicians are cautious in prescribing narcotics due to some unwanted effects including respiratory depression, nausea and vomiting, constipation, tolerance development, interaction with other drugs, and perhaps most importantly, abuse liability (Inturrisi, 2002; Bhamb *et al.*, 2006). For instance, oxycodone, fentanyl and morphine prescriptions were increased by 50, 150, and 60% between 1999 and 2002, respectively (Compton and Volkow, 2006), and during the same period, there was a 91.2% increase in deaths due to opioid poisoning (Paulozzi *et al.*, 2006). More recently, the Substance Abuse and Mental Health Services Administration (SAMHSA) national survey in 2011 showed that 4.5 million Americans used pain relievers for illicit purposes during the past month of the survey (SAMHSA, 2011). The illicit use of prescription analgesics was second only to marijuana in prevalence of illicit drug use. The same survey showed that heroin addiction almost doubled between the years 2007-2011. In 2005, the estimated cost of abusing prescription opioids in the United States was about 9.5 billion dollars (Birnbaum *et al.*, 2006). This problem also extends to other regions in the world such as Europe and Jordan (Casati *et al.*, 2012). Thus, the above statistics suggest two essential problems:

- 1) The increased use of opioids in the last 2 decades highlights the failure to find a better and safer analgesic that can replace opioids in the clinic, and

2) Science has failed to solve the secrets that underlie the mechanism of opioid addiction, and hence the abuse potential of opioids is hardly avoidable.

Thus, although much research and effort has been devoted to solve the above problems, research has so far failed to discover effective alternatives to opioid analgesics or to adequately understand the neurobiology of pain or addiction. This project proposes a new assay to measure “pain” in laboratory animals, and this may improve predictive validity with clinical results. This issue will be discussed thoroughly in section 1.3.

1.2. Opioid receptors: subtypes / signaling / mechanism of action

After the discovery of morphine and development of novel semi-synthetic and synthetic opioids, the next advance in the opioid field was the discovery of their mechanism of action. The stereospecificity of opioid effects suggested the existence of some specific sites (i.e. receptors) that could be targeted after opioid administration (Goldstein *et al.*, 1971). Using a radio-labeled opioid antagonist, the first evidence of the presence of these receptors in the nervous system was reported in 1973 (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). The presence of these receptors encouraged researchers to look for opioid-like endogenous compounds, and the first was identified in 1975 (Hughes, 1975; Hughes *et al.*, 1975). Further investigations identified three major subtypes of opioid receptors: Mu, Delta, and Kappa. These receptors were cloned in the last 2 decades (Evans *et al.*, 1992; Chen *et al.*, 1993; Meng *et al.*, 1993). A fourth type have been suggested more recently, the opioid receptor-like 1 (ORL1) receptor, and its pharmacology and significance are under

investigation (Meunier *et al.*, 1995). Further pharmacological characterization has revealed that mu opioid receptors mediate most of morphine's desired effects, such as analgesia, as well as undesired effects, such as abuse potential. One goal of the current project is to better understand the behavioral profile of mu agonist-induced abuse-related and analgesic effects, and the relationship between these effects. Characterization of this behavioral profile can then provide a foundation for research on mechanisms of those effects and strategies to dissociate them.

Opioid receptors are classified as G-protein coupled receptors (GPCRs). As with all GPCRs, these 7-transmembrane protein receptors bind extracellularly with the receptor ligand (such as morphine), and intracellularly with a complex of proteins (G-protein) consisting of three subunits: Alpha, Beta and Gamma. The alpha subunit in turn binds to Guanosine Diphosphate (GDP) during the inactive state of the receptor (hence the letter "G" in GPCR). Upon activation of the receptor (e.g. binding of an agonist), guanosine triphosphate (GTP) replaces GDP at the guanine nucleotide binding site, and GDP dissociates from the alpha subunit. This exchange promotes dissociation of all three subunits from the receptor, and also enhances the dissociation of alpha/GTP complex from the beta/gamma complex. Upon dissociation, both complexes can activate or deactivate different downstream targets such as enzymes, proteins, and/or transcription factors. The alpha subunit has GTPase activity that hydrolyzes GTP to GDP to promote rebinding with the beta/gamma complex and the receptor to terminate signaling (for review, (Lopez-Illasaca *et al.*, 1997)). Figure 1.1 summarizes the GPCR receptor activation/deactivation cycle (adapted from <http://commons.wikimedia.org/wiki/File:GPCR-Zyklus.png>).

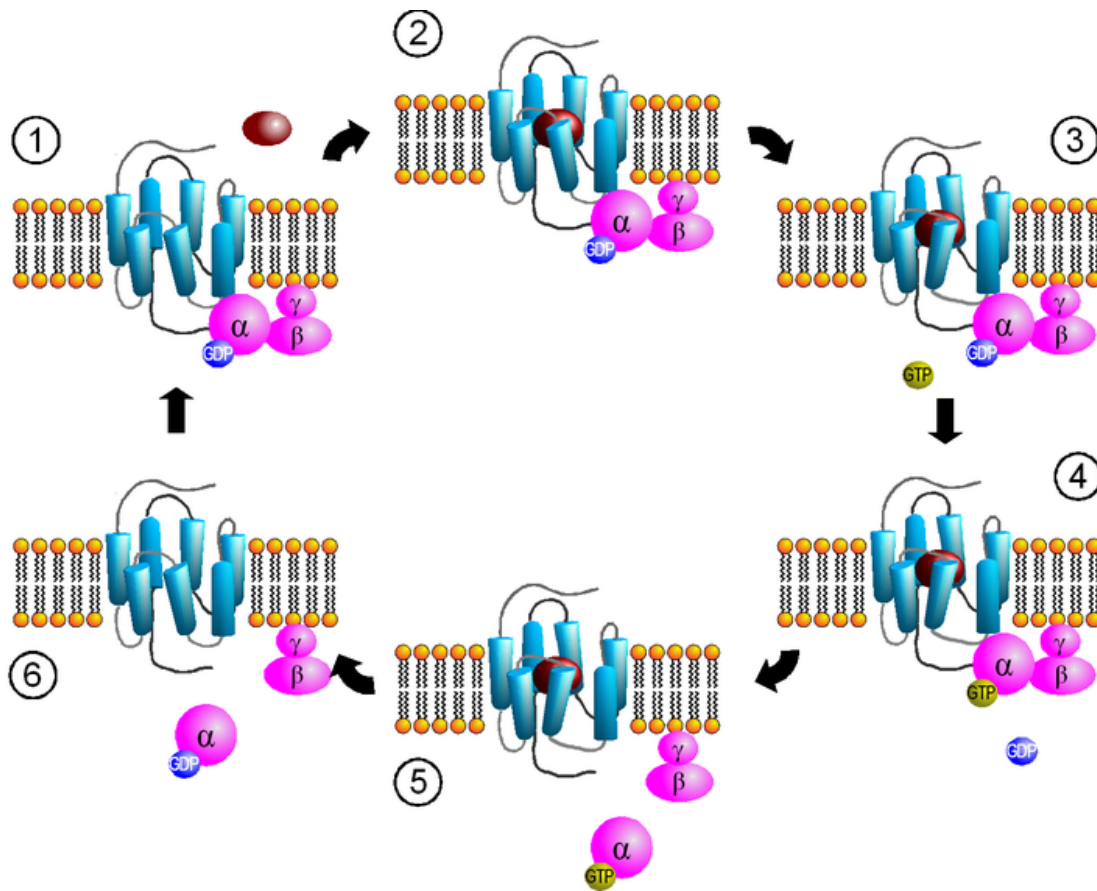


Figure 1.1. Sequence of events following the binding of a ligand to a G-protein coupled receptor. Upon binding of an agonist (step 2), Guanosine Triphosphate (GTP) replaces GDP at the guanine nucleotide binding site, and GDP dissociates from the alpha subunit (step 3 and 4). This exchange promotes dissociation of all three subunits from the receptor, and also enhances the dissociation of alpha/GTP complex from the beta/gamma complex. Upon dissociation, both complexes can activate or deactivate different downstream targets such as enzymes, proteins, and/or transcription factors. The alpha subunit has GTPase activity that hydrolyzes GTP to GDP (step 6) to promote rebinding with the beta/gamma complex (Step 1).

GPCRs are classified depending on the alpha subunit subtype. In the case of opioid receptors, they are linked to Gi/o. After a mu receptor agonist binds to the mu receptor, targets of the GTP-bound alpha subunit and free beta/gamma complex include:

- the efflux of potassium through G protein-coupled inwardly-rectifying potassium channels (GIRKs) (Lober *et al.*, 2006),
- inhibition of adenylate cyclase (and the subsequent decrease in intracellular cyclic-Adenosine Monophosphate (cAMP)) (Murthy and Makhoulf, 1996),
- inhibition of N-type voltage-dependent calcium channels (Yu *et al.*, 1990; Soldo and Moises, 1998; Ikeda *et al.*, 2000), and
- stimulation of mitogen-activated protein kinase (MAPK) pathway.

Overall, these events result in reduced neuronal cell excitability leading to a reduction in transmission of nerve impulses along with an inhibition of neurotransmitter release as summarized in figure 1.2 (McDonald and Lambert, 2005; Trescot *et al.*, 2008).

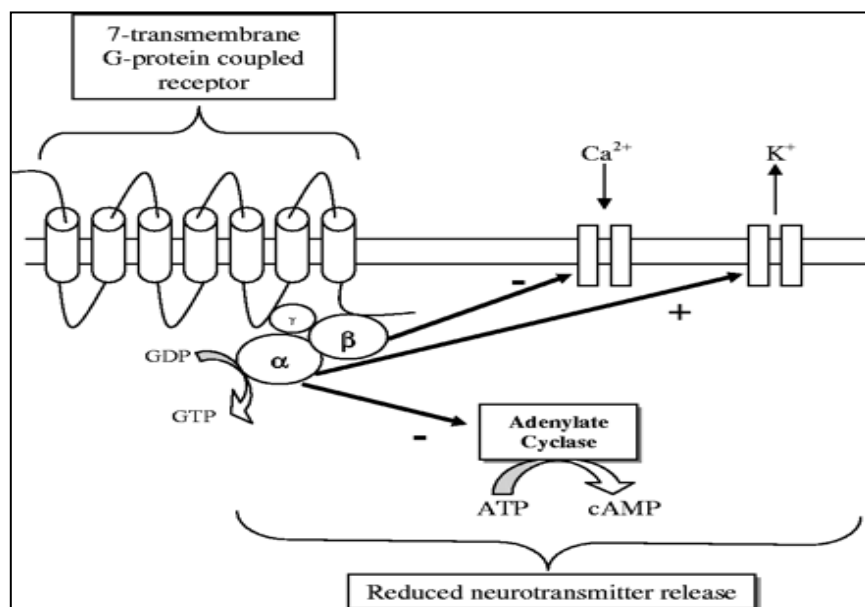


Figure 1.2. Summary of downstream signaling and second messengers coupled to an active GPCR/Gi/o. (McDonald and Lambert, 2005)

Repeated drug administration (such as daily administration of morphine) has been associated with two processes involving neural adaptation: tolerance and sensitization. Tolerance is a term used to describe the need for an increasing dose to achieve the same effect (Nestler, 1996). Possible mechanisms that contribute to opioid tolerance include receptor desensitization/internalization. Both processes may be involved in opioid tolerance after acute (i.e. after single opioid injection) or repeated opioid administration. The molecular mechanism for desensitization starts with phosphorylation of the mu receptor by G-protein receptor kinase (GRK), followed by recruitment of beta-arrestin, which uncouples the receptor from its downstream signaling cascade (Gainetdinov *et al.*, 2004; DeWire *et al.*, 2007; Nagi and Pineyro, 2011). Under some circumstances (depending on drug dose and drug efficacy at the receptor), these events of receptor phosphorylation and beta-arrestin binding may lead eventually to receptor internalization (or downregulation) (Borgland *et al.*, 2003). With both desensitization and downregulation, the density of “functional” opioid receptor is decreased, and that will diminish opioid effects, although receptor internalization may also permit recycling of functional receptors back into the membrane. Figure 1.3 summarizes receptor desensitization and internalization after binding of a ligand to GPCR receptor.

The other phenomenon that is also associated with repeated opioid administration is behavioral sensitization. It involves a progressive and enduring enhancement in the motor stimulant effect elicited by a subsequent drug challenge (Vanderschuren and Kalivas, 2000). Mechanisms of this phenomenon may include cellular adaptations in specific neural populations such as up-regulation of AMPA

receptor subunit GluR1 in the VTA (Fitzgerald *et al.*, 1996; Carlezon *et al.*, 1997) and induction of delta-FosB in the NA and amygdala (Zachariou *et al.*, 2006; Kaplan *et al.*, 2011)

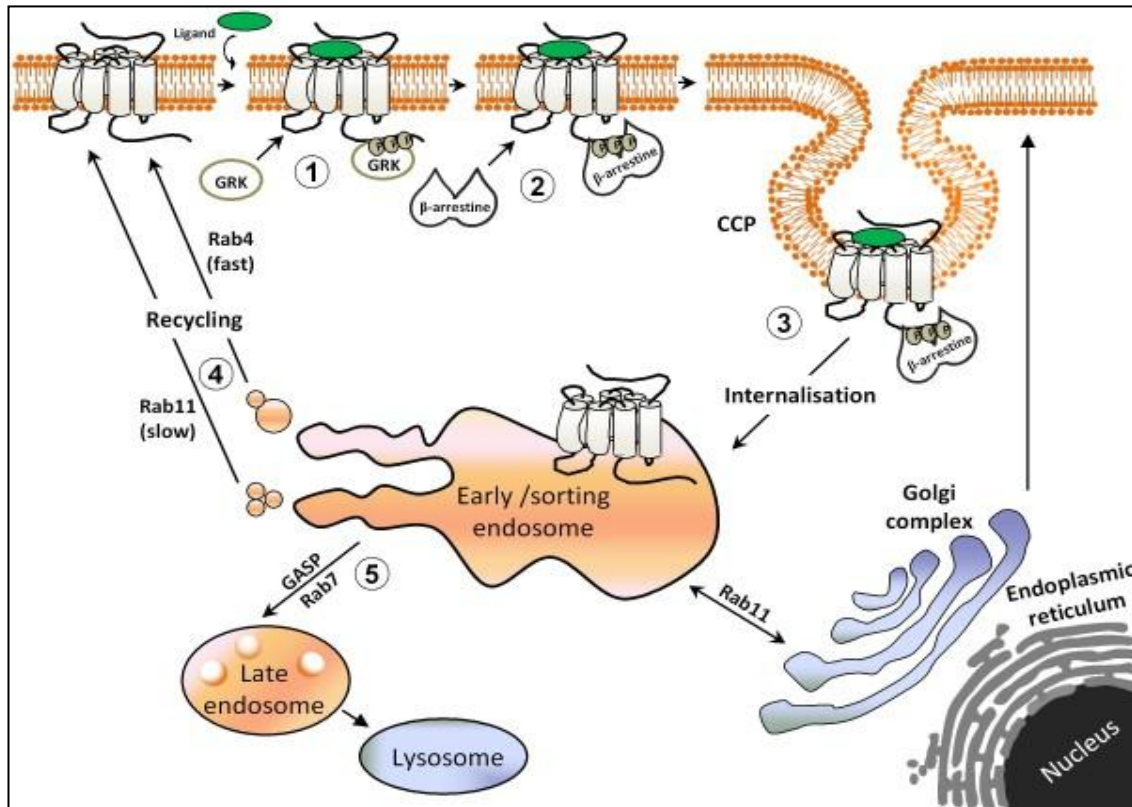


Figure 1.3. Sequence of events for desensitization and internalization of mu-opioid receptor after binding of an agonist to the receptor. (adapted from (Borgland *et al.*, 2003).

Mu opioid receptors have been localized extensively throughout the brain. Some of these areas include nucleus accumbens (NA), periaquiductal gray (PAG), ventral tegmental area (VTA), rostromedial medulla (RVM), amygdala, and thalamus (Mansour *et al.*, 1987; Dilts and Kalivas, 1989; Mansour *et al.*, 1994; Pinto *et al.*, 2008). Although mu receptors have been identified in other locations, the above brain areas are essential for analgesic and rewarding effects of opioids. One procedure to study these effects is through direct administration of the agonist into a specific brain region. For instance, administration of mu agonists into RVM, PAG, VTA, or amygdala was sufficient to induce antinociception in preclinical assays of pain-stimulated behavior (discussed later) as their dependent measure (Bodnar *et al.*, 1988; Manning *et al.*, 1994; Borszcz, 1995; Helmstetter *et al.*, 1995; Fields, 2000; Hurley *et al.*, 2003). Likewise, direct administration of mu agonists into NA or VTA was sufficient to maintain opioid self-administration in animals (Olds, 1982; Welzl *et al.*, 1989; Devine and Wise, 1994). Also, local injection of mu agonists in the VTA (Phillips and LePiane, 1980; Zangen *et al.*, 2002), but not into the NA (Olmstead and Franklin, 1997), produced conditioned place preference (CPP), which is an assay used to measure abuse-related drug reward in animals. These findings suggest that antinociceptive and abuse-related effects of mu agonists can be localized, for the most part, in separate brain areas, but they could be integrated and merged in others, such as the VTA.

So far, the VTA appears to be an especially interesting site for convergent antinociceptive and rewarding effects of mu agonists. The VTA is the site where the

mesolimbic dopaminergic neurons cell bodies are located. These neurons project to the NA, and stimulation of this pathway is often correlated with the rewarding effect of different reinforcers, such as food, sex, and drugs of abuse. In the field of opioid pharmacology, direct mu opioid agonist administration into the VTA excites dopaminergic neurons (Matthews and German, 1984). Further studies indicated that posterior VTA seems to be more relevant for mu agonist-induced reward, such that mu receptors are present in a higher density in posterior VTA than anterior VTA (Mansour *et al.*, 1995), and direct microinjection of mu agonists in the posterior VTA produced more robust acquisition of drug self-administration, conditioned place preference (CPP), and locomotor activity (Zangen *et al.*, 2002). GABAergic neurons are also present in a high density in the posterior VTA, and opioid receptors are co-localized on these inhibitory, non-dopaminergic neurons (Garzon and Pickel, 2001; Svingos *et al.*, 2001). From these findings, it has been suggested that mu opioid agonists activate dopaminergic neurons by decreasing the inhibitory influence of the caudally located GABAergic cells, a term called disinhibition (Johnson and North, 1992).

In summary, mu opioid receptors have been localized in widespread areas in the brain. These receptors are GPCRs, and they are linked to the inhibitory G-protein subtype, Gi/o. Mu opioid receptors are located on inhibitory GABAergic interneurons in the VTA, and once they bind to these cells, they attenuate GABA-mediated inhibition of dopaminergic neurons located also in the VTA. The final output is an increase in the dopaminergic neurons firing in the mesolimbic pathway, and an increase in dopamine release in the NA.

1.3. Pain: significance, neurobiology, and animal models of pain

Pain is a significant and pervasive public health problem. It has been estimated that 42% of US adults experience pain in their daily lives (Lethbridge-Cejku et al., 2004), and that this pain accounts for more than 20% of all medical visits and approximately 50 million lost work days per year (Max, 2003). The total cost to the US economy in pain-related healthcare and disability is estimated at over \$100 billion per year (Society, 2000).

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (pain, 1979). Noxious stimuli that produce tissue damage and inflammation activate primary afferent nociceptors located in the peripheral nervous system. These pseudounipolar neurons have their cell bodies in the dorsal root ganglion, with a peripheral terminal directed toward the skin, muscles, or visceral tissues, and a central terminal directed to the dorsal horn of the spinal cord. They transmit damage signals from peripheral organs to the centrally located second order neurons, also called spinothalamic neurons. These neurons have their cell bodies in the spinal cord, and after they cross the midline, they project to thalamic nuclei, where they synapse with tertiary neurons in the thalamus. Axons of the tertiary neurons project to different cortical areas such as somatosensory cortex I (SI), somatosensory cortex II (SII), anterior cingulate cortex (ACC) and insula. Limbic regions receive pain information through from two general sources. First, secondary neurons originating in the spinal cord also send bottom-up projections to brainstem areas, such as PAG, RVM, hypothalamus, and parabrachial nucleus (PBN). Some of these areas (e.g. the PBN) send projections to limbic areas such as the VTA

(Giesler *et al.*, 1979; Cliffer *et al.*, 1991; Coizet *et al.*, 2010). Second, cortical areas such as SII, ACC and insula send top-down projections to limbic areas, such as VTA, NA, and amygdala. Thus, limbic areas such as VTA, NA, and amygdala receive nociceptive input via two routes: a ventral pathway that is mediated by brain stem areas such as PBN, and a dorsal pathway that is mediated by corticolimbic connections. Moreover, neurons in the PAG send projections to the RVM and dorsolateral pontine tegmentum (DLPT), and both of them send descending projections back to the spinal cord for further modulation of nociceptive information (Mantyh and Peschanski, 1982; Basbaum and Fields, 1984). Figure 1.4 summarizes some of these connections that are of interest to the current document. These connections have consolidated the view that pain is a complex experience encompassing sensory, affective and cognitive elements (Zubieta *et al.*, 2001; Neugebauer *et al.*, 2009).

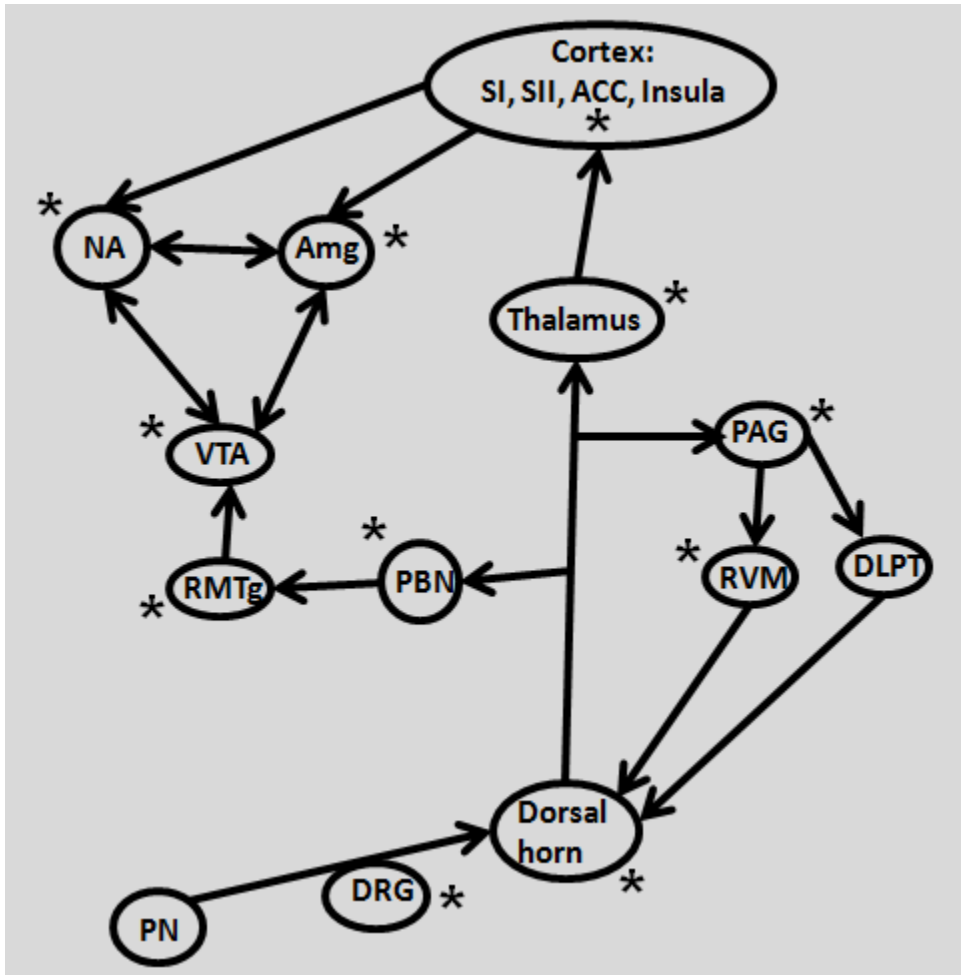


Figure 1.4. Simplified cartoon showing major pain pathways and connections between them inside the nervous system. Arrows indicate the direction of signal between regions. Some areas have reciprocal connection. Asterisks indicate that mu opioid receptors have been localized in that area. ACC: anterior cingulate cortex; Amg: Amygdala; DLPT: dorsolateral pontine nucleus; DRG: dorsal root ganglion; NA: nucleus accumbens; PAG: periaqueductal gray; PBN: parabrachial nucleus; PN: primary nociceptors; RMTg: rostromedial tegmentum; RVM: rostroventral medulla; SI, II: Sensory area 1 and 2; VTA: ventral tegmental area.

Analgesia is defined as absence of pain in response to stimulation that would normally be painful (International Association for the Study of Pain, 1994). Despite recent advances in research on pain mechanisms and analgesic drug development, there has been little evolution in the types of drugs used to treat pain. For decades, the most widely used analgesics have included mu opioid agonists such as morphine, anti-inflammatory steroids such as cortisone, and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. As mentioned before, opioids are still the drug of choice over other options to treat severe, chronic types of pain.

Pain is essentially a subjective experience, and its existence in humans is typically assessed by verbal report. This report is usually guided by a scale that ranges from 0-10, with “0” indicating “no pain” and “10” indicating “worst pain imaginable.” Alternatively, patients can complete a questionnaire that provides added verbal detail to their pain perception. An example of such a questionnaire is the Brief Pain Inventory (BPI) (Daut *et al.*, 1983), which provides information on the intensity of pain as well as the degree to which pain interferes with function. The BPI also asks questions regarding pain relief, pain quality, and patient's perception of the cause of pain.

In preclinical settings, assays of animal behavior have played a key role in research on the neurobiology of pain and development of analgesic drugs (Negus *et al.*, 2006). These preclinical assays share two common elements: (a) a set of independent variables implemented with the intent of producing a pain state (i.e. noxious stimuli), and (b) a dependent measure of behavior interpreted as evidence of that pain state. A noxious stimulus can be chemical, mechanical, thermal, or electrical in modality. In all these cases, application of the noxious stimulus is intended to produce “pain,” and

changes in behavior produced by the noxious stimulus are interpreted as evidence of “pain.” Drugs or other treatments can then be evaluated for their effects on expression of the pain-related behavior. The behavioral outcomes usually fall into one of the following two categories:

- 1) Pain-stimulated behaviors: include behaviors that increase in rate, frequency or intensity after a noxious stimulus. These include, but are not limited to, withdrawal reflexes from escapable stimuli (e.g. tail withdrawal after immersion of the tail into water heated to a noxious temperature), withdrawal-like behaviors from inescapable stimuli (e.g. stretching/writhing elicited by intraperitoneal (IP) injection of dilute acids or chemical irritants), and vocalization;
- 2) Pain-depressed behaviors: include behaviors that decrease in rate, frequency or intensity after a noxious stimulus. Examples of this type of behavior include depression of feeding, locomotion, and operant responding, sleep and others.

Antinociception in assays of pain-stimulated behavior are indicated by drug-induced decreases in the target behavior. However, exclusive reliance on pain-stimulated behaviors to evaluate effects of opioids or other candidate analgesics is problematic for several reasons (Negus *et al.*, 2006). Perhaps most importantly, drug-induced decreases in pain-stimulated behavior can be produced not only by a selective reduction in sensory sensitivity to the noxious stimulus (i.e. true analgesia) but also by nonselective effects such as motor impairment (resulting in “false positive” effects). Another drawback of relying on pain-stimulated behaviors is the lack of face validity of these behaviors with problematic dimensions of clinical pain. Noxious stimuli can elicit

withdrawal reflexes and other pain-stimulated behaviors in humans, and there are clinical instances (e.g. surgery) in which it is desirable to reduce these behaviors. However, clinical suppression of pain-stimulated behaviors is usually managed with transient exposure to anesthetics, and long-term suppression of pain-stimulated behaviors can increase risk of injury by preventing adaptive withdrawal responses from noxious stimuli. Clinically problematic pain, by comparison, is often expressed as functional impairment and depression of behavior and mood, and the goal of treatment is to normalize function and mood. As mentioned earlier, the Brief Pain Inventory is one instrument that measures the impact of pain conditions on quality of life, such as general activity, mood, walking ability, normal work, sleep, and social activity. Most of these behaviors are often depressed during clinically relevant pain conditions, and effective analgesics will reverse this depression.

In contrast to pain-stimulated behaviors, antinociception in assays of pain-depressed behavior is indicated by an increase in the target behavior, and as a result, these assays are not vulnerable to false-positive effects of drugs that produce motor impairment. Assays of pain-depressed behavior may also add value in analgesic drug development for two other reasons. First, the diagnosis of pain in both human and veterinary medicine often relies on measures of pain-depressed behavior (also referred to as “functional impairment”), and restoration of pain-depressed behavior is often a goal of treatment (Cleeland and Ryan, 1994; Dworkin *et al.*, 2005). The utility of these measures in clinical contexts suggests that pain-depressed behaviors may also be useful as endpoints in research. Second, pain-related depression of behavior is often accompanied by comorbid depression of mood in humans (Bair *et al.*, 2003; Maletic and

Raison, 2009), and preclinical research on pain-depressed behavior may provide insights into the expression, neurobiology and modulation of the affective dimensions of pain. Thus, assays of pain-depressed behavior would serve as a better tool to evaluate candidate analgesics in the laboratory animals and would produce better translational pain research to the clinical field.

Decreases in locomotion, feeding, and positively reinforced operant responding are common examples of pain-depressed behaviors in laboratory animals. Operant conditioning provides one strategy for generating high, stable and quantifiable rates of baseline behavior that can be used to assess effects of putative pain states. Intracranial self-stimulation (ICSS) in rats is one example of operant responding. In this procedure, rats are implanted with intracranial electrodes targeting the medial forebrain bundle, and lever-press responding is maintained under a schedule of brain stimulation. The ability of brain stimulation to function as a reinforcing stimulus was first discovered in 1954 (Olds and Milner, 1954), and ICSS maintained by stimulation of the medial forebrain bundle is mediated by activation of excitatory inputs to the mesolimbic dopamine neurons that originate in VTA. Electrical stimulation to the medial forebrain bundle, and the subsequent activation of dopaminergic neurons in the mesolimbic pathway, functions as a highly reinforcing stimulus compared to other reinforcers such as food. Moreover, it generates a stable behavior that can be efficiently assessed during short daily behavioral sessions, and it is non-satiable compared to behaviors maintained by other reinforcers such as food. Also, using ICSS gives the experimenter the ability to determine the anatomical significance of brain areas in pain and/or analgesia by directly implanting the electrode into the area(s) of interest in the animal's brain. In this case,

the brain of area of interest is the VTA given its potential role in both abuse-related and analgesic effects of mu agonists. Finally, it has been shown that administration of an acute noxious stimulus, such as intraperitoneal injection of lactic acid, depresses ICSS, and this depression can be blocked by pretreatment with clinically effective analgesics such as NSAIDs. Thus, for all these reasons, this project uses ICSS as a behavioral baseline for research on pain-depressed behavior that will be beneficial to examine the pharmacology of opioids in the presence and absence of noxious stimulus.

1.3.1 Antinociceptive effects of opioids in assays of pain-stimulated behavior

Pain-stimulated behaviors are widely used in animal research to evaluate drug-induced antinociception. As one simple example, tail-withdrawal assays of thermal nociception apply a noxious thermal stimulus (e.g. a hot light or hot water) to the tail of a rodent or non-human primate and measure the latency to a tail withdrawal response. In this example, application of the hot stimulus is intended to produce “pain,” and tail withdrawal is an unconditioned behavioral response interpreted as evidence of “pain”. The intensity of the noxious stimulus (hot water) is often manipulated by simply changing the temperature of the hot water. Another example to induce “pain” in animals is by injecting them with a chemical irritant, such as intraperitoneal injection of dilute acid. This will produce withdrawal-like stretching/writhing from an inescapable stimulus (in this case, acid). In both examples, drugs or other treatments can then be evaluated for their effects on expression of the pain-related behavior.

Opioids have been extensively studied in these and other assays of pain-stimulated behavior, and opioids consistently produced antinociception in these assays.

For example, pretreatment with morphine produced thermal antinociception in a warm-water tail-withdrawal assay in rats and mice (Morgan *et al.*, 1999; Cook *et al.*, 2000), and decreased the number of stretches after IP administration of acid in mice and rats (Barbaz *et al.*, 1988; Pereira Do Carmo *et al.*, 2009). These assays are used also to determine the role of drug efficacy at the mu opioid receptor. For example, low-efficacy mu agonists such as nalbuphine and buprenorphine often produce antinociception against low- but not high-intensity noxious thermal stimuli, whereas higher efficacy mu agonists such as morphine, fentanyl and methadone are more likely to produce antinociception against both low- and high-intensity noxious stimuli (Morgan *et al.*, 1999; Negus and Mello, 1999; Cook *et al.*, 2000). Such data provide one source of evidence to suggest that antinociception against low-intensity thermal noxious stimuli has lower efficacy requirements than antinociception against high-intensity thermal noxious stimuli. Likewise, high- and low-efficacy mu agonists produced a decrease in stretching response after IP administration of acid (Pchelintsev *et al.*, 1991; Patrick *et al.*, 1999). However, to the author's knowledge, the interaction between drug efficacy at the mu receptor and noxious stimulus intensity has not been investigated previously in assays of chemically-induced stretching. The author expects that high-efficacy agonists will retain their antinociceptive effectiveness at high- and low- stimulus intensities, whereas low-efficacy agonists will lose their antinociceptive effects at the high stimulus intensity.

In addition to these effects of mu opioid efficacy, antinociception in assays of pain-stimulated behavior is also modified by regimens of prior opioid exposure. For example, repeated morphine administration produced tolerance to morphine's

antinociception effects in both a warm-water tail-withdrawal assay (Walker and Young, 2001) and in an assay of acid-stimulated stretching (Taber *et al.*, 1969).

The goal of this project was to extend this preclinical assessment of opioid antinociception to an assay of pain-depressed behavior. In particular, experiments in this project will explore the degree to which mu agonist antinociception in an assay of pain-depressed ICSS is determined by the efficacy of the mu agonist and by the degree of prior opioid exposure.

1.4. Intracranial self-stimulation and opioids

As a complement to assessment of mu agonists' effects on ICSS in the presence of pain, we also examined effects of mu agonists on ICSS in the absence of pain. As mentioned earlier, ICSS comprises a family of operant procedures in which responding is maintained by electrical stimuli delivered to target brain regions such as the medial forebrain bundle at the level of the lateral hypothalamus (Olds and Milner, 1954; Carlezon and Chartoff, 2007). One application of ICSS has been to generate stable baselines of schedule controlled responding for use in evaluating abuse-related drug effects (Kornetsky *et al.*, 1979; Wise, 1998). Within this research tradition, facilitation of ICSS (indicated by increased rates of ICSS) is often interpreted as a rewarding drug effect that may contribute to, or be predictive of, abuse liability by that drug in humans. For example, amphetamine is representative of one class of abused drugs (i.e. indirect dopamine agonists) that reliably and robustly facilitate ICSS across a broad range of experimental conditions in rats (Esposito *et al.*, 1980; Do Carmo *et al.*, 2009).

Mu opioid agonists, including morphine, constitute another class of abused drugs that has been evaluated extensively in ICSS, but mu agonist effects on ICSS have been less consistent than effects produced by amphetamine-like stimulants. More specifically, mu agonist effects seem to be influenced by the particular type of procedure used to assess ICSS performance, and by other factors that include pretreatment time and extent of prior exposure to opioids. In the simplest type of ICSS procedure, responding produces electrical stimulation of a constant magnitude, and the primary dependent measure is the rate of responding or reinforcement. This approach can generate relatively constant rates of ICSS, and early studies using this approach revealed two major findings (Adams *et al.*, 1972; Lorens and Mitchell, 1973; Koob *et al.*, 1975). First, acute treatment with morphine or other mu agonists produced effects with a biphasic time course consisting of an initial decrease followed by a subsequent increase in rates of ICSS. Second, repeated or chronic treatment produced tolerance to the initial rate-decreasing effects and earlier expression of rate-increasing effects. As appreciated by investigators using this constant-reinforcer magnitude approach, expression of rate-decreasing or rate-increasing effects depended in part on baseline ICSS rates engendered by the selected reinforcer magnitude. High stimulus magnitudes (i.e. high intensity or frequency) maintained high ICSS rates preferentially sensitive to rate-decreasing effects, whereas lower stimulus magnitudes maintained lower ICSS rates preferentially sensitive to rate-increasing effects.

More recent studies have evaluated mu agonist's effects using more sophisticated ICSS procedures, in which reinforcer magnitude is systematically manipulated during each experimental session by manipulating either the intensity or

the frequency of electrical stimulation. Although these procedures have the potential to efficiently assess drug effects on a wide range of ICSS rates maintained by a wide range of reinforcer magnitudes, the focus has been on threshold reinforcer magnitudes that maintain low rates or low probabilities of responding and are especially sensitive to rate-increasing effects linked to abuse liability. In accordance with this sensitivity, some studies reported rapid facilitation of ICSS after acute treatment with low mu agonist doses (Kornetsky and Esposito, 1979; Carlezon and Wise, 1993; Jha *et al.*, 2004), although this finding has not always been obtained (Stratmann and Craft, 1997; Pereira Do Carmo *et al.*, 2009). Other studies have provided evidence to suggest that, in accordance with earlier studies using simpler procedures, facilitation of ICSS may be more robust later in the time course after acute mu agonist treatment or after chronic treatment (Craft *et al.*, 2001; O'Neill and Todtenkopf, 2010).

In the current project, the effect of mu opioid agonist on ICSS in the presence and in the absence of pain had been evaluated using a 'frequency–rate' procedure, in which the frequency of the reinforcing electrical stimulus was varied to generate a wide range of response rates during each daily session.

1.5. Introduction to data chapters

The goal of this project was to assess opioid pharmacology in ICSS as an assay of pain-depressed behavior. As mentioned earlier, ICSS is a sensitive assay to the abuse-related effects of drugs of abuse, including opioids. Thus, this project evaluated opioid agonists in two phases:

1) Phase one: effects of mu opioids in the absence of noxious stimulus. During this phase, mu opioid agonists were tested in ICSS in conjunction with manipulation of different independent variables, including pretreatment time, dose, drug efficacy at the mu receptor, and repeated drug administration. Results of these studies are presented and discussed in three sections (chapters):

- a) Chapter 2: Effects of morphine dose, pretreatment time, repeated treatment, and rate-dependency on ICSS. Morphine is the prototype opioid analgesic to which all other opioids are compared. It is the first opioid to be discovered and isolated from the poppy plant.
- b) Chapter 3: Effects of methadone and nalbuphine on ICSS. Methadone is a high-efficacy mu opioid ligand, while nalbuphine is a low-efficacy ligand at the mu receptors. The efficacy of these drugs was determined previously using agonist-stimulated GTP Gamma [S] binding assay (Selley *et al.*, 1998). Morphine`s efficacy at the mu receptor falls between that of methadone and nalbuphine.
- c) Chapter 4: Effects of high-, moderate-, and low-efficacy mu opioid agonists on ICSS before, during, and after chronic morphine exposure. Methadone, fentanyl, and nalbuphine (from high to low efficacy at the mu receptor) were tested on ICSS before morphine exposure when subjects were opioid naive, during 3.2 mg/kg/day morphine exposure, and during 18 mg/kg/day morphine exposure. The doses of morphine were selected to produce different degrees of tolerance and dependence, depending on results from the previous sections. Tolerance is a term used to describe

the need for an increasing dose to achieve the same effect; while dependence describes an altered physiological state caused by repeated drug exposure such that cessation of drug administration (or after administration of an antagonist) leads to a withdrawal syndrome (Nestler, 1996). Dependence was evaluated using the mu-opioid receptor antagonist, naltrexone.

2) Phase 2: effects of mu opioid agonists in assays of acid-stimulated stretching and acid-induced depression of ICSS. The same drugs used during phase one were tested again in ICSS as a pretreatment to a noxious stimulus. The noxious stimulus used in these experiments was intraperitoneal injection of dilute lactic acid. Lactic acid releases protons that activate C-fibers through the activation of Transient Receptor Potential Vanilloid 1 (TRPV1) receptors (Julius and Basbaum, 2001; Tang *et al.*, 2007), as well as acid-sensing ion channels (Shimada *et al.*, 2004). An assay of acid-stimulated stretching was used as a comparison to acid-depressed ICSS. Two sections summarize results from this phase:

a) Chapter 5: effects of mu opioid agonists on acid-stimulated stretching and acid-depressed ICSS: role of mu agonist efficacy and noxious stimulus intensity. Methadone, fentanyl, morphine, hydrocodone, buprenorphine, and nalbuphine (from high to low efficacy at the mu receptor (Selley *et al.*, 1997; Selley *et al.*, 1998; Thompson *et al.*, 2004)) were tested in both assays as a pretreatment to 1.8% lactic acid. Methadone and nalbuphine effects on ICSS were determined

during noxious stimulus intensity manipulation, and were tested as a pretreatment to 5.6% lactic acid.

- b) Chapter 6: Morphine antinociception during repeated morphine administration in assays of acid-stimulated stretching and acid-induced depression of ICSS. A chronic morphine regimen was used to assess tolerance to the antinociceptive effects of morphine in both assays. The regimen described in chapter 6 was chosen based on pilot experiments. The control group received a daily injection of saline.

CHAPTER TWO

Some determinants of morphine effects on intracranial self-stimulation in rats: dose, pretreatment time, repeated treatment, and rate dependence

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2.1. Introduction

Mu agonist effects on ICSS have been less consistent than effects produced by other classes of abused drugs, such as stimulants. One reason behind this inconsistency may be related to the type of procedure used to assess ICSS performance. More specifically, the most commonly used procedures to assess opioid's effects on ICSS are simple rate procedures and threshold determination procedures. One disadvantage of using simple rate procedures is that drug effects on ICSS are highly dependent on baseline rate of responding. Threshold determination procedures overcome this disadvantage; However, the focus has been on threshold reinforcer magnitudes that maintain low rates or low probabilities of responding and are especially sensitive to rate-increasing effects linked to abuse liability. Other factors that determine opioid effects on ICSS include pretreatment time and history of drug administration.

The main goal of this study was to further evaluate the role of dose, pretreatment time, and regimen of repeated treatment as determinants of morphine effects on ICSS using a 'frequency–rate' procedure, in which the frequency of the reinforcing electrical stimulus was varied to generate a wide range of response rates during each daily session.

2.2. Methods

2.2.1. Subjects

Ten adult male Sprague–Dawley rats (Harlan, Frederick, Maryland, USA) weighing 310–350 g at the time of surgery were used. Rats were individually housed and were maintained on a 12 h light/dark cycle, with lights on from 06:00 to 18:00 h. Rats had free access to food and water except during testing. Subject maintenance and research were in compliance with National Institutes of Health guidelines on care and use of subjects in research, and all subject-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

2.2.2. Assay of intracranial self-stimulation

Intracranial self-stimulation electrode implantation. Rats were anesthetized with isoflurane gas (2.5–3% in oxygen; Webster Veterinary, Phoenix, Arizona, USA) for implantation of stainless steel electrodes (Plastics One, Roanoke, Virginia, USA). One pole (the cathode) of each bipolar electrode was 0.25mm in diameter and covered with polyamide insulation except at the flattened tip, whereas the other pole (the anode) was

0.125mm in diameter and uninsulated. The cathode was implanted in the left medial forebrain bundle at the level of the lateral hypothalamus (2.8mm posterior to bregma, 1.7mm lateral from midsagittal suture, and 7.8mm below dura). The anode was wrapped around one of three skull screws to serve as the ground, and the skull screws and electrode assembly were secured to the skull with orthodontic resin. The subjects were allowed to recover for at least 7 days before commencing ICSS training.

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

Behavioral procedure. After initial shaping of lever-press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures similar to those described previously (Do Carmo *et al.*, 2009a, 2009b; Negus *et al.*, 2010). During experimental sessions, each lever press resulted in the delivery of a 0.5-s train of square wave cathodal pulses (0.1 ms pulse duration), and stimulation was accompanied by the illumination of the stimulus lights over the lever. Responses during the 0.5 s stimulation period did not earn additional stimulation. During initial training sessions lasting 30–60 min, the frequency of stimulation was held

constant at 158 Hz, and the stimulation intensity for each rat was adjusted gradually to the lowest value that would sustain a high rate of reinforcement (>30 stimulations/min). Once this criterion was met, frequency manipulations were introduced. Sessions involving frequency manipulations consisted of sequential 10-min components. During each component, a descending series of 10 current frequencies (158–56 Hz in 0.05 log increments) was presented, with a 60 s trial at each frequency. A frequency trial was initiated by a 5 s time-out followed by a 5 s 'priming' phase, during which subjects received five noncontingent stimulations with a 0.5 s interval between each stimulation. This noncontingent stimulation was then followed by a 50 s 'response' phase, during which responding produced electrical stimulation under the continuous reinforcement schedule. Training continued with presentation of up to three sequential components per day, and the current intensity was again adjusted at this stage of training until rats reliably responded for the first three to four frequency trials of all components for at least three consecutive days. This intensity was then held constant for the remainder of the study.

Acute-dosing study. Once training was completed, subsequent studies examined (a) the time course of acute morphine doses and (b) the effects of repeated morphine doses. Test sessions to examine the time course of acute morphine doses consisted of multiple 10-min components identical to those described above. Each session began with three consecutive 'baseline' components. The first baseline component 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

section Data analysis). Morphine (1.0–10.0mg/kg) or its vehicle (saline) was administered immediately after the third baseline component. Subsequently, consecutive pairs of test components were initiated 10, 30, 100, and 300min after morphine or vehicle treatment. Thus, ICSS performance was evaluated 10–30, 30–50, 100–120, and 300–320min after each treatment. Morphine doses were delivered in a mixed order across rats. Test sessions were typically conducted on Tuesdays and Fridays and were separated by at least 3 days. Training sessions consisting of three components were conducted on Mondays, Wednesdays, Thursdays, and occasionally on Saturdays. In some cases, data from these training sessions were used to assess ICSS performance 24 h after high doses of morphine. In these cases, data from the second and third components of the training session were used as the ‘24 h time point’ for data analysis.

Chronic-dosing study. At the conclusion of the acute-dosing study, the effects of repeated morphine were evaluated. During the first 3 days of this phase of the study, rats were exposed daily to three consecutive ‘baseline’ components. Data from the first component each day were discarded, and data for the second and third components of each day were averaged to generate the baseline frequency–rate curve for comparison with subsequent chronic morphine effects (see section Data analysis). Immediately after the third baseline component on the third day, repeated morphine treatments were initiated for a period of four consecutive weeks. Rats were treated with 3.2 mg/kg/day morphine during week 1, 5.6 mg/kg/day morphine during week 2, 10mg/kg/day morphine during week 3, and 18mg/kg/day morphine during week 4. This regimen dose was used to minimize lethal outcomes after high morphine doses. Morphine doses were

administered daily as a single bolus injection at 1:30 p.m., and rats were exposed to two consecutive ICSS test components 30min after each morphine dose. The following day, 23.5 h after each morphine dose (and immediately before the next morphine dose), rats were exposed to another three consecutive ICSS test components, and data from the second and third of these components were used for data analysis. Thus, ICSS performance was evaluated 30min and 23.5 h after each morphine dose. After the last dose of 18 mg/kg/day morphine, morphine dosing was terminated, and three-component test sessions were conducted daily for an additional 3 days. Again, data from the second and third components of these sessions were used for data analysis to assess changes in ICSS during the first 3 days of withdrawal. The primary goal of this study was to quantify the effects of chronic morphine treatment and withdrawal on ICSS. However, to provide some assessment of potential somatic withdrawal signs, rats were also weighed daily, cages were inspected for evidence of diarrhea, and subjects were observed for signs of teeth chattering and wet-dog shakes immediately before each daily session.

2.2.3. Drugs

Morphine sulfate was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland, USA). All solutions were prepared in sterile water for subcutaneous injection.

2.2.4. Data Analysis

The primary dependent variable in this ICSS procedure was the reinforcement rate in stimulations/min during each frequency trial. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percent maximum control rate (%MCR) for that rat. During the acute-dosing study, the MCR was determined daily and was defined as the mean of the maximal rates observed in any frequency trial during the second and third 'baseline' components for that day. For the repeated-dosing study, the MCR was determined before the initiation of repeated morphine dosing and was defined as the mean of the maximal rates observed during the second and third 'baseline' components over a period of three consecutive days (six total baseline components). Thus, %MCR values for each trial were calculated as $(\text{Response Rate during a Frequency Trial} / \text{MCR}) \times 100$.

For the acute-dosing study, data from each pair of consecutive test components at each time point after morphine injection were averaged and normalized to the MCR for that day as discussed above. For the repeated dosing study, data from each pair of test components 30 min and 23.5 h after injection were averaged and normalized to the MCR determined before the initiation of the study as discussed above. For statistical analysis, normalized data were compared by two-way analysis of variance (ANOVA), with treatment time and ICSS frequency as the two factors. A significant ANOVA was followed by a Holm–Sidak post-hoc test, and the criterion for significance was set at P value of less than 0.05.

To provide an additional summary of ICSS performance during the repeated-dosing study, the total number of stimulations per component was calculated as the

sum of stimulations delivered across all 10 frequency trials of each component. Test data were then normalized to Morphine effects on ICSS baseline data using the equation $\% \text{ Baseline Stimulations per Component} = (\text{Mean Stimulations per Test Component} / \text{Mean Stimulations per Baseline Component}) \times 100$. Data were then averaged across rats under each experimental condition and compared by one-way ANOVA. A significant ANOVA was followed by the Dunnett post-hoc test, and the criterion for significance was set a priori at P value of less than 0.05.

Finally, rate-dependent effects of morphine under selected conditions were examined by graphing log percent control ICSS rate after morphine treatment as a function of log control ICSS rate for each stimulation frequency. For the purposes of rate-dependency analysis, control ICSS rates at each brain stimulation frequency were defined as the mean number of stimulations obtained at each frequency after vehicle treatment for the acute studies, and during the baseline sessions before initiation of chronic morphine in the chronic studies. Percent control reinforcement rates after morphine were calculated as $[(\text{number of stimulations after morphine} / \text{number of control stimulations}) \times 100]$ for each stimulation frequency. The analysis was applied to data collected (a) 30 and 100 min after acute treatment with 3.2, 5.6, and 10 mg/kg morphine, and (b) 30 min after the seventh daily treatment with chronic 3.2, 5.6, and 10 mg/kg morphine. The resulting rate-dependency plots were subjected to linear regression analysis using Prism 5 for Macintosh (GraphPad Software Inc., La Jolla, California, USA). Morphine effects were considered to be significantly rate-dependent if the 95% confidence limits of the slope did not include '0' and if P value of less than 0.05 for the regression.

2.3. Results

2.3.1. Effects of acute morphine

For each test session, a 'baseline' ICSS frequency–rate curve was determined before testing to permit the determination of the MCR for that session. During studies of acute morphine effects, the average MCR was 56.3 ± 13.0 stimulations/trial.

Reinforcement rates for each rat during each frequency trial of a session were then normalized as the %MCR for that rat in that session, and the average baseline frequency–rate curves for each test are shown in Figure 2.1 (gray lines, open squares). Rats generally did not respond at frequencies of 56–79 Hz, and reinforcement rates increased across a frequency range of 112–158 Hz. Maximum reinforcement rates were usually observed at the highest stimulation frequencies. There were no statistically significant differences between baselines on different days (data not shown).

Figure 2.1 also shows the time course of effects produced by vehicle and morphine (1.0–10 mg/kg), and detailed statistical results are provided in the figure legend. Vehicle injection had little effect on ICSS frequency–rate curves, producing significant but only small increases in reinforcement rates at 100 Hz at 10 and 100 min after treatment. Morphine produced dose-dependent, time-dependent, and frequency-dependent changes in ICSS. At earlier time points (10–30 min), the predominant effect of morphine was a dose-dependent decrease in reinforcement rates maintained by high frequencies of brain stimulation (112–158 Hz). These rate-decreasing effects peaked at 30 min, and the highest dose of 10 mg/kg morphine nearly eliminated responding at 30 min. In addition to these predominant rate-decreasing effects at 10 and 30 min, low

doses of 1.0 and 3.2 mg/kg morphine also produced significant but small increases in reinforcement rates at some lower frequencies. After 100 min, the rate-decreasing effects of morphine had dissipated for all but the highest morphine dose, and doses of 3.2 and 5.6mg/kg morphine produced significant and robust rate-increasing effects at intermediate frequencies of brain stimulation (71–100Hz), resulting in leftward shifts in frequency–rate curves relative to baseline. After 300min, none of the morphine doses produced effects significantly different from baseline.

Overall, these results suggested that morphine produced rate-decreasing effects with a relatively short duration of action and rate-increasing effects with a longer duration of action. Data with 10 mg/kg morphine suggested that a period of predominant rate-increasing effects may have been missed between 100 and 300 min. To evaluate this possibility, effects of morphine were determined 180 min after administration of 10 mg/kg morphine in a separate group of three rats (Fig. 2.2). These results confirmed that 10 mg/kg produced a robust facilitation of ICSS at this time point.

2.3.2. Effects of chronic morphine

Figure 2.3 shows effects of chronic morphine on ICSS. Each panel shows the baseline frequency–rate curve determined before chronic treatment together with the frequency–rate curves determined 30 min after morphine on the first and seventh day of treatment with each morphine dose (3.2–18mg/kg/day). Thus, chronic studies evaluated morphine effects at a time (30 min) when initial acute studies revealed primarily rate-decreasing effects of morphine.

Before chronic treatment, the MCR was 54.0 ± 14.1 stimulations/trial, and the baseline frequency–rate curve was similar to that described above. Lower doses of 3.2 and 5.6 mg/kg morphine produced only rate-increasing effects and leftward shifts in frequency–rate curves relative to baseline on both days 1 and 7 of treatment. In general, there was little difference in the effects of morphine on days 1 and 7, although for the initial dose of 3.2 mg/kg morphine, response rates were slightly but significantly higher at frequencies of 70 and 112 Hz on day 7 as compared with day 1. The higher two doses of 10 and 18 mg/kg morphine produced only rate-decreasing effects on the first day of treatment, and these rate-decreasing effects were greater for 18 mg/kg than for 10 mg/kg morphine. However, after 7 days of treatment, these rate-decreasing effects were no longer apparent, and both doses produced only rate-increasing effects.

To assess the impact of morphine abstinence and spontaneous morphine withdrawal on ICSS, frequency–rate curves were also determined before each daily morphine injection (i.e. 23.5 h after the injection on the previous day). This period of abstinence was associated with a dose-dependent decrease in ICSS. For example, figure 2.4a shows summary data for ICSS 23.5 h after the last injection of each dose, and during the 3 days after termination of treatment with the highest dose of 18 mg/kg/day morphine. After 3.2 and 5.6 mg/kg morphine, there were slight but non-significant decreases in the total number of stimulations delivered. This effect, however, was significant after 10 and 18 mg/kg daily morphine administration. Figure 2.4b shows the frequency–rate curve 23.5 h after the last dose of 18 mg/kg morphine. ICSS recovered completely back to baseline levels within 3 days after termination of treatment with 18 mg/kg morphine. Rats were also observed for signs of somatic

withdrawal before daily ICSS sessions and for 3 days after termination of chronic morphine. Chronic morphine produced a dose-dependent decrease in body weight. From a mean \pm SEM starting weight before chronic treatment of 454.2 ± 15.7 g, morphine produced mean \pm SEM body weight losses of 1.6 ± 1.1 , 2.3 ± 1.1 , 4.4 ± 0.9 , and $7.2 \pm 1.0\%$ after 3.2, 5.6, 10, and 18mg/kg morphine, respectively. However, this decrease in body weight could not confidently be attributed to withdrawal as distinct from a direct morphine effect, and other somatic signs of opioid withdrawal (diarrhea, teeth chattering, and wetdog shakes) were not observed.

2.3.3. Rate-dependent effects of morphine on intracranial self-stimulation

Figure 2.5 shows the degree to which morphine effects on ICSS varied as a function of baseline ICSS rates. Data are shown for results obtained 30min after acute or chronic treatment with 3.2, 5.6, and 10mg/kg morphine to illustrate the breadth of changes in rate dependency. Table 2.1 shows results of linear regression analysis applied to the rate-dependency plots shown in Figure 5, and also shows data obtained 100 min after acute administration of each dose. Overall, the extent of rate dependency was influenced by dose, pretreatment time, and chronicity of treatment. Effects of the lowest dose of 3.2mg/kg were not rate-dependent 30 or 100min after acute administration, but effects were rate-dependent 30min after the last dose of chronic treatment. Effects of the intermediate dose of 5.6mg/kg morphine were not rate-dependent 30min after acute treatment, but became rate-dependent 100min after acute treatment and 30min after the last dose of chronic treatment. Effects of the high dose of 10 mg/kg morphine were rate-dependent under all conditions; however, the nature of

that rate dependency changed. Thus, acute 10 mg/kg morphine had little or no effect on low baseline rates of ICSS but decreased high baseline rates of ICSS. Conversely, after chronic administration, morphine increased low baseline rates of ICSS but had little effect on high baseline rates. Overall, morphine exposure produced either by the longer 100min pretreatment time or by chronic treatment had the general effect of increasing the negative slope, correlation coefficient, and statistical significance of rate-dependency plots and shifting these plots vertically upward. After chronic treatment, all morphine doses produced significant rate-dependent effects expressed as an increase in low baseline rates of ICSS and little change in high baseline rates of ICSS.

2.3.4. Summary

This study examined morphine effects on ICSS in rats using a ‘frequency–rate’ procedure, in which a wide range of ICSS rates was maintained by a wide range of brain stimulation frequencies during each daily session. There were three main findings. First, acute morphine produced time-dependent changes in ICSS such that rate-decreasing effects predominated at earlier time points (10–30 min) whereas rate-increasing effects predominated at later time points (100–180 min). Second, repeated morphine produced tolerance to rate-decreasing effects and unmasked robust rate-increasing and rate-dependent effects 30 min after morphine administration. Finally, withdrawal from repeated morphine produced small but significant decreases in ICSS that recovered over the course of 3 days after withdrawal from the highest morphine dose. Taken together, these data indicate that morphine dose and pretreatment time,

the history of morphine exposure, and baseline ICSS rate are critical determinants of both the magnitude and the valence of morphine effects on ICSS.

Figure 2.1

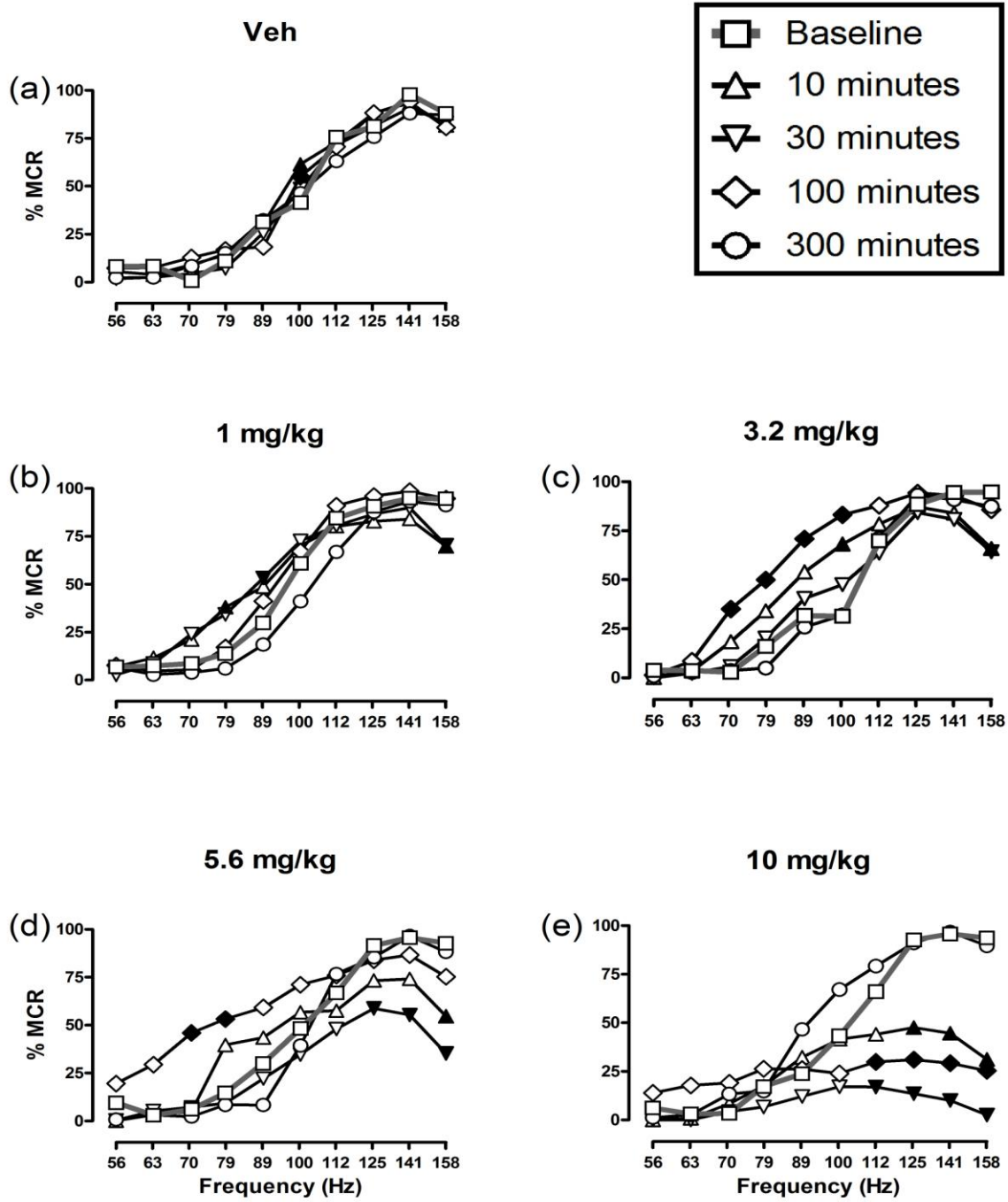


Figure 2.1. Morphine pretreatment produced dose-dependent, time-dependent, and frequency-dependent changes in intracranial self-stimulation (ICSS).

Horizontal axes: frequency of electrical brain stimulation in hertz (log scale). Vertical axes: ICSS rate expressed as percent maximum control rate (%MCR). (a–e) shows ICSS frequency–rate curves determined before (baseline) or at various times after (10–300 min) treatment with vehicle (Veh) or morphine (1.0–10 mg/kg). Filled symbols indicate frequencies at which ICSS rates were significantly lower or higher than baseline as determined by the Holm–Sidak post-hoc test following a significant analysis of variance (ANOVA) ($P < 0.05$). ANOVA results were as follows: Vehicle: significant main effect of frequency [$F(9,45)=44.0$; $P < 0.001$], significant main effect of time [$F(4,20)=3.5$; $P=0.025$], no significant frequency x time interaction [$F(36,180) = 1.4$; NS]; 1 mg/kg morphine: significant main effect of frequency [$F(9,45)=148.0$; $P < 0.001$], no significant main effect of time [$F(4,20)=1.6$; NS], significant frequency x time interaction [$F(36,180) = 3.0$; $P < 0.001$]; 3.2 mg/kg morphine: significant main effect of frequency [$F(9,45)=98.7$; $P < 0.001$], significant main effect of time [$F(4,20)=3.9$; $P < 0.02$], significant frequency x time interaction [$F(36,180) = 2.5$; $P < 0.001$]; 5.6 mg/kg morphine: significant main effect of frequency [$F(9,45)=88.1$; $P < 0.001$], significant main effect of time [$F(4,20)=4.2$; $P < 0.02$], significant frequency x time interaction [$F(36,180) = 4.0$; $P < 0.001$]; 10 mg/kg: significant main effect of frequency [$F(9,45)=45.4$; $P < 0.001$], significant main effect of time [$F(4,20)=4.0$; $P < 0.02$], significant frequency x time interaction [$F(36,180) = 9.0$; $P < 0.001$]. All points show mean data for six rats, and error bars are omitted for clarity.

Figure 2.2

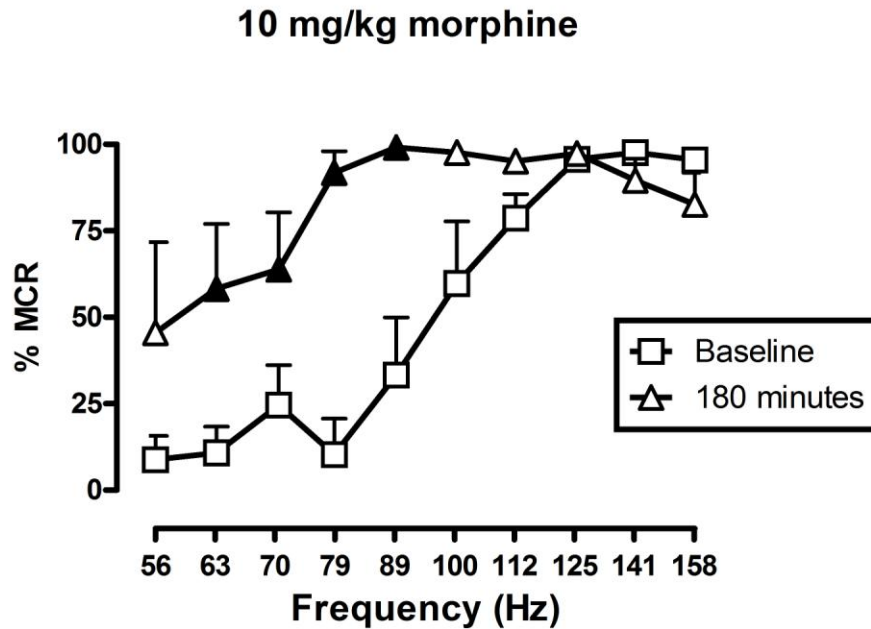


Figure 2.2. 10 mg/kg Morphine facilitated intracranial self-stimulation (ICSS) after 180 min. Horizontal axis: frequency of electrical brain stimulation in hertz (log scale). Vertical axis: ICSS rate expressed as percent maximum control rate (%MCR). Filled symbols indicate frequencies at which ICSS rates were significantly higher than baseline as determined by the Holm–Sidak post-hoc test following a significant analysis of variance (ANOVA) ($P < 0.05$). ANOVA revealed a significant main effect of frequency [$F(9,18)=17.3$; $P < 0.001$], no significant effect of treatment [$F(1,2)=9.9$; $P=0.09$], but a significant frequency x treatment interaction [$F(9,18)=4.0$; $P < 0.01$]. All points show mean data for three rats.

Figure 2.3

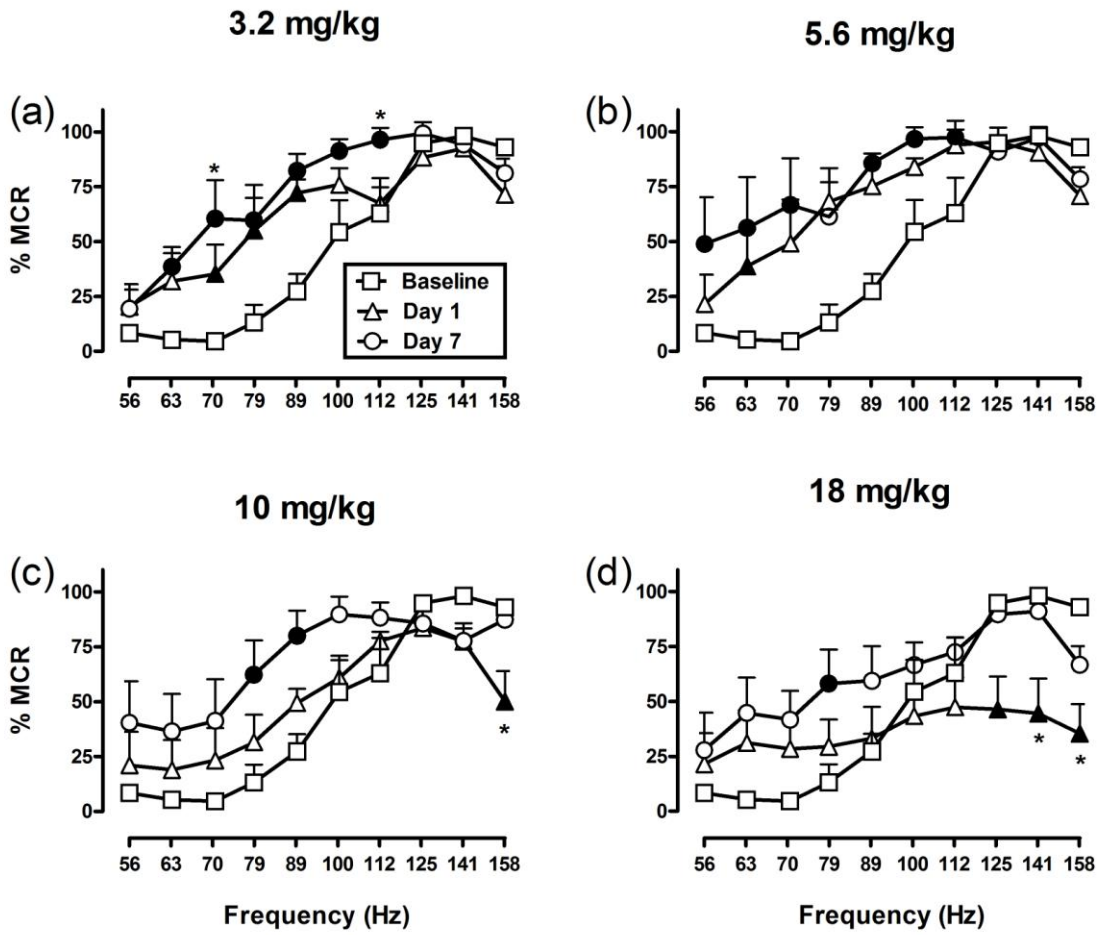


Figure 2.3. Effects of chronic morphine on intracranial self-stimulation (ICSS).

Horizontal axes: frequency of electrical brain stimulation in hertz (log scale). Vertical axes: ICSS rate expressed as percent maximum control rate (%MCR). Rats were treated for 28 consecutive days with an ascending sequence of four morphine doses (3.2, 5.6, 10, and 18 mg/kg/day). Each dose was administered for 7 days, and ICSS frequency–rate curves were determined 30 min after each injection. (a–d) shows frequency–rate data obtained before chronic morphine (baseline) and on the first and seventh days of treatment with each morphine dose. Filled symbols indicate frequencies at which ICSS rates were significantly lower or higher than baseline, and asterisks indicate frequencies at which ICSS rates on day 7 were significantly higher than rates on day 1, as determined by the Holm–Sidak post-hoc test following a significant analysis of variance (ANOVA) ($P < 0.05$). ANOVA results were as follows: 3.2 morphine: significant main effect of frequency [$F(9,36) = 28.2$; $P < 0.001$], significant main effect of day [$F(2,8) = 5.3$; $P < 0.05$], significant frequency x day interaction [$F(18,72) = 4.4$; $P < 0.001$]; 5.6 morphine: significant main effect of frequency [$F(9,36) = 18.5$; $P < 0.001$], significant main effect of day [$F(2,8) = 4.5$; $P < 0.05$], significant frequency x day interaction [$F(18,72) = 4.5$; $P < 0.001$]; 10 morphine: significant main effect of frequency [$F(9,36) = 25.2$; $P < 0.001$], no significant main effect of day [$F(2,8) = 3.0$; NS], and a significant frequency x day interaction [$F(18,72) = 3.2$; $P < 0.001$]; 18 morphine: significant main effect of frequency [$F(9,36) = 27.6$; $P < 0.001$], no significant main effect of day [$F(2,8) = 2.2$; NS], and a significant frequency x day interaction [$F(18,72) = 5.9$; $P < 0.001$]. All points show mean data for five rats.

Figure 2.4

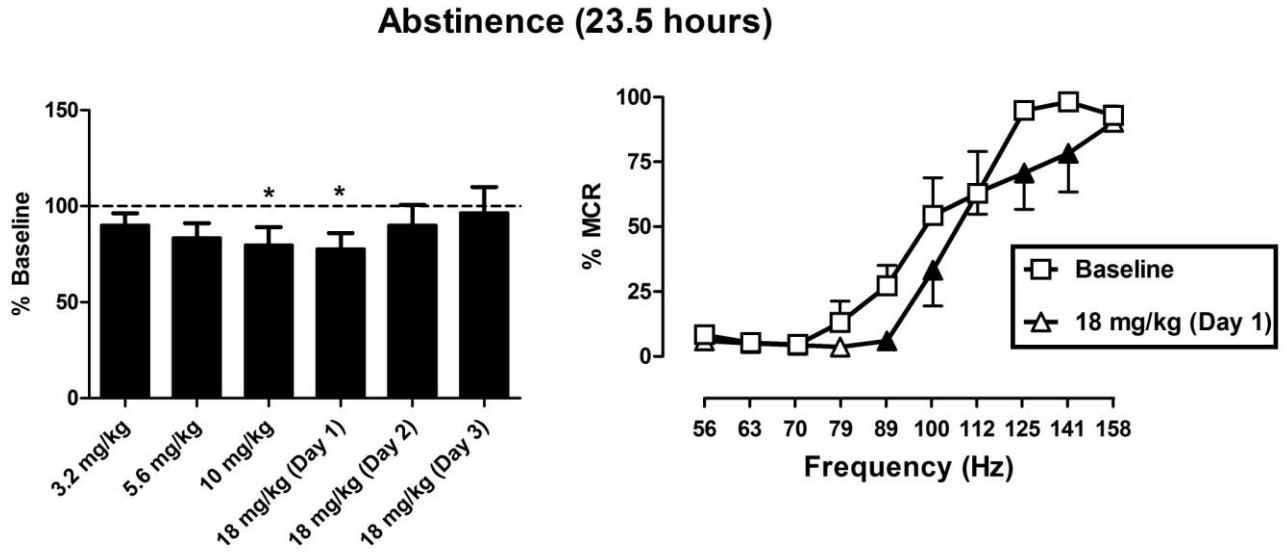


Figure 2.4. Effects of morphine abstinence on intracranial self-stimulation (ICSS).

(a) shows the total number of stimulations per component expressed as a percentage of baseline stimulations per component 23.5 h after the last injection of each dose and during the 3 days after the last dose of 18 mg/kg morphine. Horizontal axis: dose of morphine (mg/kg) before abstinence. Vertical axis: percent baseline number of stimulations per component. One-way analysis of variance (ANOVA) indicated a significant main effect of abstinence condition [$F(6,24)=3.13$; $P<0.025$]. Asterisks indicate conditions under which total number of stimulations was significantly lower than baseline (100%), as determined by the Dunnett post-hoc test. (b) shows the baseline frequency–rate curve and the frequency–rate curve determined on the day after the last dose of 18 mg/kg morphine. Horizontal axis: frequency of brain stimulation in hertz (log scale). Vertical axis: ICSS rate expressed as percent maximum control response rate (%MCR). Two-way ANOVA indicated a significant main effect of frequency [$F(9,36)=57.1$; $P<0.001$], significant main effect of day [$F(1,4)=8.3$; $P<0.05$], but no significant frequency x day interaction [$F(9,36)=1.3$; NS]. Filled symbols indicate frequencies at which reinforcement rates were significantly lower than baseline as determined by the Holm–Sidak post-hoc test. All bars and symbols show mean data from five rats, and error bars in the right panel show SEM

Figure 2.5

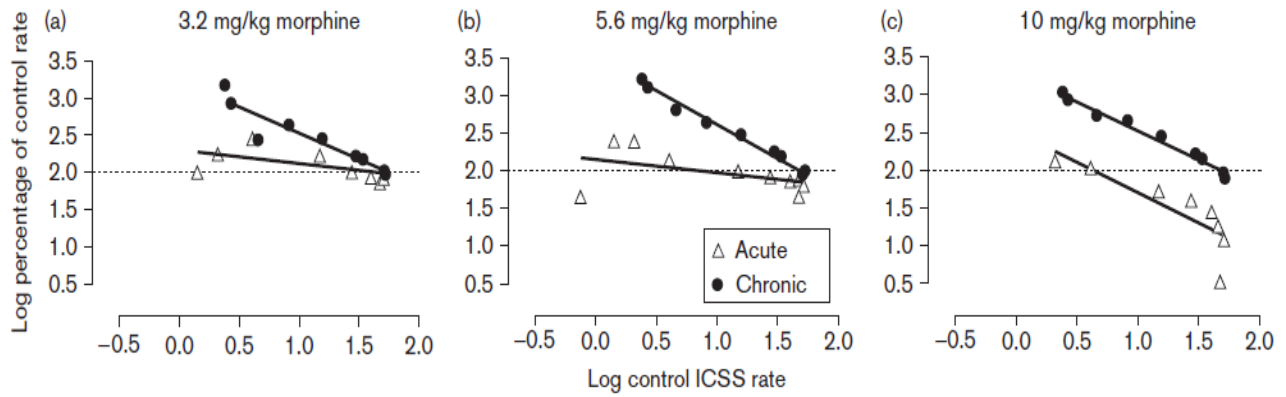


Figure 2.5. Rate dependency of morphine effects on intracranial self-stimulation (ICSS). Horizontal axes: Log control ICSS rate (in units of stimulations/ frequency trial) at each of the 10 frequencies of brain stimulation. Vertical axes: log percent control ICSS rate observed 30 min after morphine treatment. (a–c) show effects of 3.2, 5.6, and 10 mg/kg morphine during the acute-dosing phase of the study, and after the seventh daily dose during the chronic dosing phase of the study. All points show mean data for five to six rats.

Table 2.1. Linear regression analysis applied to the rate-dependency plots shown in Fig. 5 and to data obtained 100 min after acute administration of each dose

Morphine dose (mg/kg)	Acute vs. chronic	Slope	95% confidence interval	<i>P</i> value	<i>R</i> ²
3.2	Acute (30 min)	-0.18	-0.40 to +0.04	0.09	0.36
	Acute (100 min)	0.04	-0.65 to +0.73	0.89	0.00
	Chronic ^a	-0.73	-0.95 to -0.52	<0.01	0.89
5.6	Acute (30 min)	-0.18	-0.45 to +0.08	0.15	0.24
	Acute (100 min) ^a	-0.60	-0.79 to -0.42	<0.01	0.88
	Chronic ^a	-0.87	-0.95 to -0.79	<0.01	0.99
10	Acute (30 min) ^a	-0.81	-1.35 to -0.28	0.01	0.70
	Acute (100 min) ^a	-0.80	-0.92 to -0.76	<0.01	0.97
	Chronic ^a	-0.78	-0.86 to -0.71	<0.01	0.99

^aTreatment conditions at which 95% confidence intervals of the slope did not include '0' and *P*<0.05 for the regression.

CHAPTER THREE

Role of mu-agonist efficacy as determinants of the effects of the mu agonists on intracranial self-stimulation in rats

(2012; *Behavioural Pharmacology* 23(7):678-92)

3.1. Introduction

One possible explanation of my findings in chapter two is that mu agonist-induced facilitation of ICSS requires lower levels of receptor occupation and activation (i.e. has lower efficacy requirements and higher receptor reserve) than mu agonist-induced depression of ICSS. According to this hypothesis, lower morphine doses would produce sufficient receptor occupancy and activation to facilitate ICSS, whereas higher doses would produce higher levels of receptor occupancy sufficient to recruit opposing rate-decreasing effects. Repeated morphine could selectively attenuate rate-decreasing effects and enhance expression of rate-increasing effects by desensitizing and/or downregulating some fraction of mu receptors to reduce the functional receptor density and attenuate the ability of morphine to produce rate-decreasing effects dependent on high levels of receptor occupancy (Martini and Whistler, 2007). The present study tested this hypothesis by testing the effect of a high- and a low- efficacy agonists on ICSS. The hypothesis predicted that higher-efficacy mu agonists would have sufficient efficacy to

produce both morphine-like rate-increasing and rate-decreasing effects, but that lower efficacy mu agonists might lack sufficient efficacy to produce rate-decreasing effects and might therefore produce enhanced rate-increasing effects.

3.2. Methods

3.2.1. Subjects

Subjects are similar to those described on section 2.2.1. The total number of subjects in this study was 17.

3.2.2. Assay of intracranial self-stimulation

Intracranial self-stimulation electrode implantation and behavioral procedure are similar to those described on section 2.2.2.

Effects of opioid agonists with varying efficacies at the mu receptor. Three groups of rats were used in this experiment. In the first group, the high efficacy agonist methadone (0.032–5.6 mg/kg), the low efficacy agonist nalbuphine (0.032–10 mg/kg), naltrexone (0.1 mg/kg), and vehicle (saline) were tested (n=5). Morphine was tested first, and the results were similar to those reported previously (Altarifi and Negus, 2011), and are not discussed further. For each drug, except naltrexone, testing was conducted in two phases. During the first phase, the dose was manipulated from doses that produced no effect on ICSS to doses that either (a) significantly decreased ICSS or (b) were at least 10 times greater than the lowest dose to significantly facilitate ICSS. Test sessions consisted of three consecutive control components, followed immediately by

injection of the drug and then 30 min later by two consecutive test components. During the second phase, the time course of the dose producing peak facilitation of ICSS was determined. Test sessions consisted of three consecutive control components, followed immediately by injection of the drug and then by consecutive pairs of test components that began 10, 30, 100, 180, and 300 min after injection. Naltrexone was tested at a single dose (0.1 mg/kg) alone and as a 20-min pretreatment to 1.0 mg/kg methadone. One rat died before studies with naltrexone; thus, these studies were carried out with only four rats. The remaining two groups were used in experiments to examine the effects of the highest efficacy mu agonist methadone (0.032–3.2 mg/kg) and the lowest efficacy mu agonist nalbuphine (0.032–10 mg/kg) in separate groups of opioid-naive rats.

In all experiments, sessions were typically conducted on Tuesdays and Fridays and were separated by at least 3 days. This intermittent-dosing regimen was intended to minimize the development of tolerance to drug effects on ICSS. Training sessions consisting of three components were conducted on Mondays, Wednesdays, Thursdays, and occasionally, on Saturdays. In some cases, data from these training sessions were used to assess ICSS performance 24 h after injection in the time-course studies. In these cases, data from the second and the third components of the training session were used as the '24-h time point' for data analysis.

3.2.3. Drugs

Methadone HCl and naltrexone HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland, USA). Nalbuphine HCl was

provided by Dr Kenner Rice (Chemical Biology Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland, USA). All drugs were dissolved in saline and delivered subcutaneously in a volume of 1ml/kg body weight.

3.2.4. Data analysis

The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial were converted into the percent maximum control rate (%MCR) for that rat on that day. The MCR was determined during the control components of each test session and was defined as the mean of the maximal rates observed in any frequency trial during the second and third control components. Thus, %MCR for each trial was calculated as (reinforcement rate during a frequency trial / MCR) x 100. Normalized data from the frequency trials of each pair of consecutive test components were then averaged across rats for display and for statistical analysis using two-way analysis of variance (ANOVA), with drug dose or time as one factor and ICSS frequency as the other factor. A significant ANOVA was followed by a Holm–Sidak post-hoc test, and the criterion for significance was set at P less than 0.05.

To provide an additional summary of ICSS performance, the total number of stimulations per component was calculated as the average of the total stimulations delivered across all 10 frequency trials of each component. Test data were expressed as a percentage of the total stimulations earned during the 'control' components (% control stimulations). Thus, % control stimulations for each test were calculated as

(mean total stimulations during test components / mean total stimulations during control components) x 100.

3.3. Results

The control parameters of ICSS did not vary significantly during sequential testing with mu-opioid receptor ligands in a group of five rats, and the overall mean control parameters of all three groups are shown in Table 3.1. In the first group (i.e. has history of opioid administration), the high efficacy mu agonist methadone exerted dose-dependent effects on ICSS, manifested as the exclusive facilitation of ICSS at relatively low to intermediate doses (0.32 - 1.0 mg/kg) and emergence of rate-decreasing effects at high doses (3.2 – 5.6 mg/kg; Fig. 3.1). The lower efficacy mu agonist nalbuphine also produced exclusive facilitation of ICSS at relatively low doses; however, in contrast to the higher efficacy mu agonist methadone, nalbuphine continued to produce exclusive facilitation of ICSS at doses up to 30-fold higher than the lowest doses to produce facilitation (Fig. 3.1). The opioid antagonist naltrexone did not alter ICSS at a dose sufficient to antagonize methadone-induced facilitation of ICSS (Fig. 3.1). Both drugs had the same potency to facilitate ICSS and figure 3.2 shows that both drugs produced peak facilitation of ICSS at the earliest time tested (10 min), and the speed of offset was faster for nalbuphine than methadone (100 vs. 180 min, respectively). Treatment with vehicle (saline) did not produce significant changes in ICSS at any time point.

Figure 3.3 shows the effects of methadone and nalbuphine in two separate groups of opioid-naive rats. Relative to their effects in the initial group of opioid-

experienced rats shown in Figure 3.1, methadone and nalbuphine produced weaker ICSS facilitation, and methadone produced more potent ICSS depression. For example, 1.0mg/kg methadone facilitated low rates of ICSS maintained by low frequencies of brain stimulation in opioid-experienced rats (Fig. 3.1), but only depressed high rates of ICSS maintained by high frequencies of brain stimulation in opioid-naive rats (Fig. 3.3). Similarly, nalbuphine produced greater magnitudes of ICSS facilitation across a broader range of frequencies in opioid-experienced rats (Fig. 3.1) than in opioid-naive rats (facilitation only at 89Hz after 0.32 and 10 mg/kg; Fig. 3.3). Notably, nalbuphine did not significantly decrease ICSS at any dose in opioid-naive rats, but this absence of rate-decreasing effects was not associated with enhanced expression of rate-increasing effects relative to methadone.

3.4. Summary

Mu opioid receptor agonists such as morphine can either facilitate or depress ICSS, and previous opioid exposure can increase the expression of abuse-related ICSS facilitation (Chapter 2). This study tested the hypothesis that rate-decreasing effects require higher activation of mu receptors, and hence are more vulnerable to tolerance-associated reductions in receptor density, than rate-increasing effects. I tested a high efficacy mu agonist (methadone) as well as a low efficacy mu agonist (nalbuphine). Each drug was tested twice in two separate groups of subjects: the first group was opioid experienced, and the other group was naïve. The hypothesis predicted that nalbuphine, would mimic the effects of morphine tolerance to produce the reduced expression of rate-decreasing effects and enhanced expression of rate-increasing

effects, while methadone would mimic the effects of morphine during acute administration. In contrast to this hypothesis, in opioid-naïve rats, a reduction in the efficacy of mu agonists was associated with a decreased expression of rate-decreasing effects, but not a tolerance-like enhancement in ICSS facilitation. These results suggest that history of opioid exposure influences effects of mu opioid receptor agonists on ICSS, and that mu agonist-induced facilitation and depression of ICSS may be mediated by distinct populations of mu receptors that respond differently to regimens of opioid exposure.

Figure 3.1.

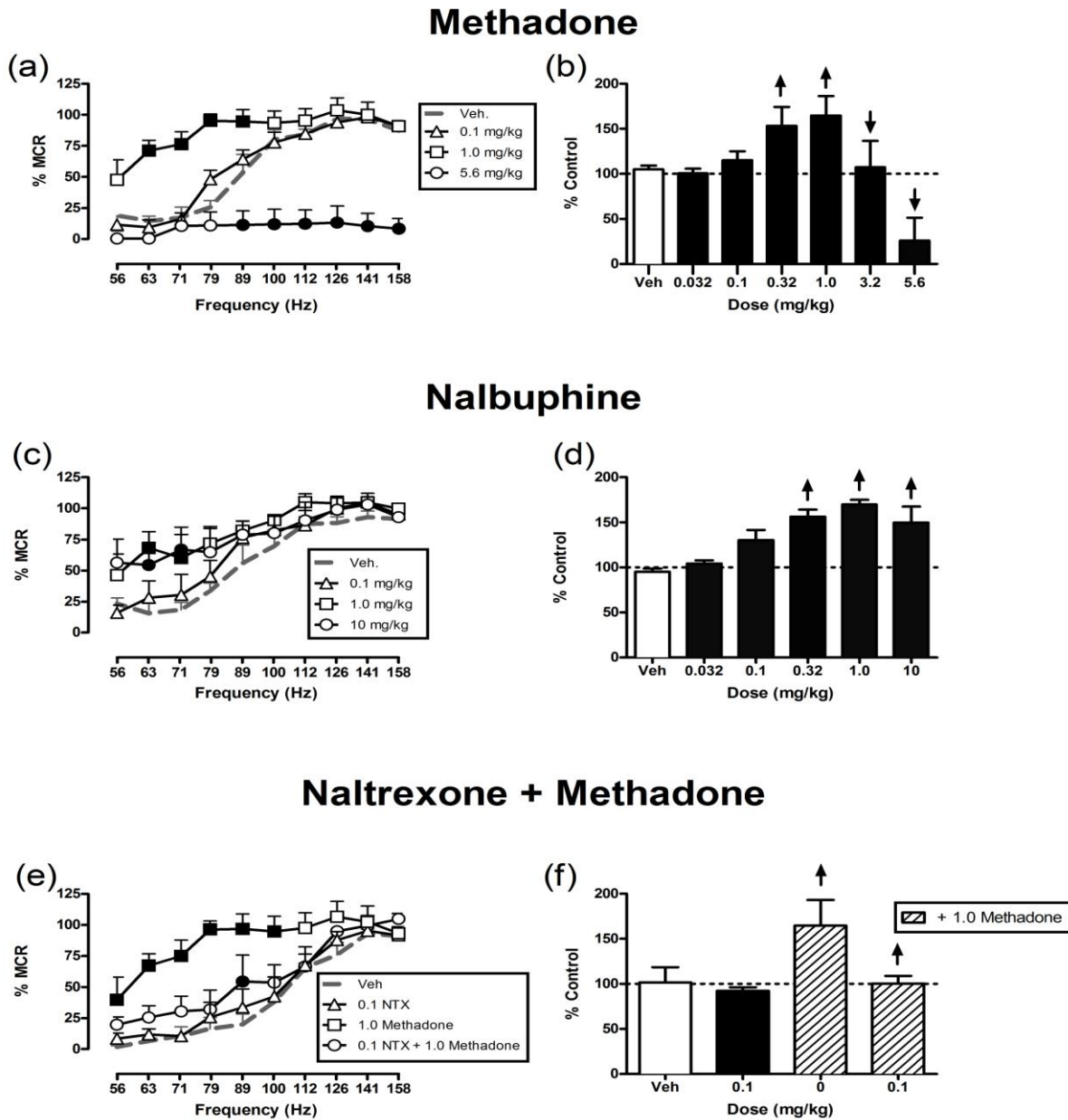


Figure 3.1. Effects of methadone (a, b), nalbuphine (c, d), or “naltrexone + methadone” (e, f) on ICSS in opioid-experienced rats. The left column (panels a, c, and e) shows the ICSS frequency–rate curves. Horizontal axes: frequency of electrical brain stimulation in Hz (log scale). Vertical axes: ICSS rate expressed as percent maximum control rate (%MCR). Data obtained for some doses were excluded from the graph for clarity, but were included in statistical analyses. Filled symbols indicate the frequencies at which morphine ICSS rates were greater than those obtained during control components, as determined by the Holm–Sidak post-hoc test following a significant two-way ANOVA. The right column (panels b, d, and f) shows the total number of stimulations per test component, expressed as a percentage of the total control stimulations. Horizontal axes: dose. Vertical axes: percent control stimulations per test component. Upward and downward arrows indicate the presence and valence of the effects of test drug as determined by analyses of frequency–rate data. Thus, upward arrows indicate significant facilitation of ICSS at ≥ 1 frequency of the frequency–rate curve, whereas downward arrows indicate significant depression of ICSS at ≥ 1 frequency of the frequency–rate curve. ANOVA results were as follows: methadone: significant main effect of frequency [$F(9,36) = 71.2$; $P < 0.001$], significant main effect of dose [$F(5,20) = 3.8$; $P = 0.014$], and significant dose x frequency interaction [$F(45,180) = 3.8$; $P < 0.001$]; nalbuphine: significant main effect of frequency [$F(9,36) = 27.1$; $P < 0.001$], significant main effect of dose [$F(5,20) = 10.8$; $P < 0.001$], and significant dose x frequency interaction [$F(45,180) = 1.8$; $P = 0.005$]; naltrexone: significant main effect of frequency [$F(9,27) = 27.4$; $P < 0.001$], significant main effect of treatment [$F(3,9) = 7.1$; $P = 0.009$], and significant treatment x frequency interaction [$F(27,81) = 3.5$; $P < 0.001$]. ANOVA, analysis of variance; ICSS, intracranial self-stimulation; MCR, maximum control rate; NTX, naltrexone; Veh, vehicle

Figure 3.2.

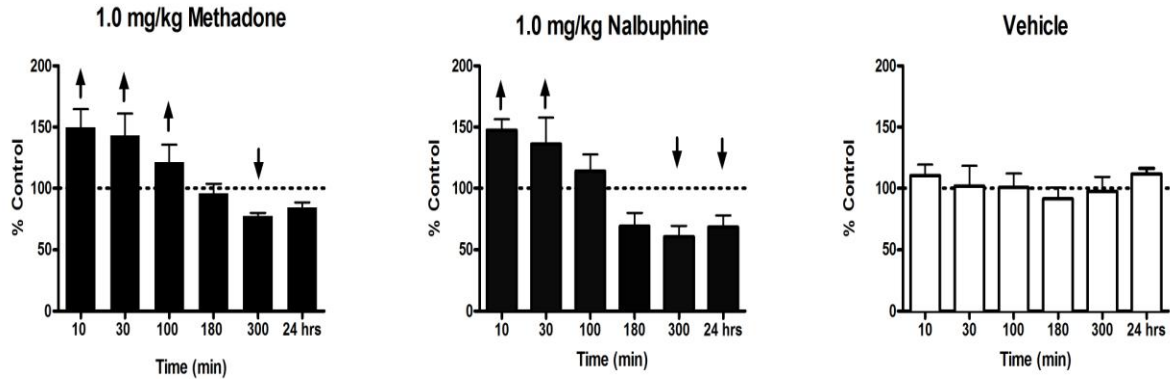


Figure 3.2. Time courses of mu-opioid receptor agonist effects on ICSS. Time course of the dose producing peak facilitation of ICSS for each drug as shown in Figs 3.1 was determined. Horizontal axes: time elapsed after the injection of the test drug. Vertical axes: percent control stimulations per test component. ANOVA results were as follows: methadone: significant main effect of frequency [$F(9,36) = 92.8$; $P < 0.001$], significant main effect of time [$F(6,24) = 15.1$; $P < 0.001$], and significant time x frequency interaction [$F(45,216) = 2.2$; $P < 0.001$]; nalbuphine: significant main effect of frequency [$F(9,27) = 36.5$; $P < 0.001$], significant main effect of time [$F(5,15) = 7.8$; $P < 0.001$], and no significant time x frequency interaction [$F(45,135) = 1.2$; $P = 0.181$]; vehicle: significant main effect of frequency [$F(9,27) = 36.7$; $P < 0.001$], no significant main effect of time [$F(6,18) = 1.6$; $P = 0.215$], and no significant time x frequency interaction [$F(54,162) = 1.2$; $P = 0.250$]. For a description of symbols, please refer to Fig. 1. ANOVA, analysis of variance; ICSS, intracranial self-stimulation.

Figure 3.3

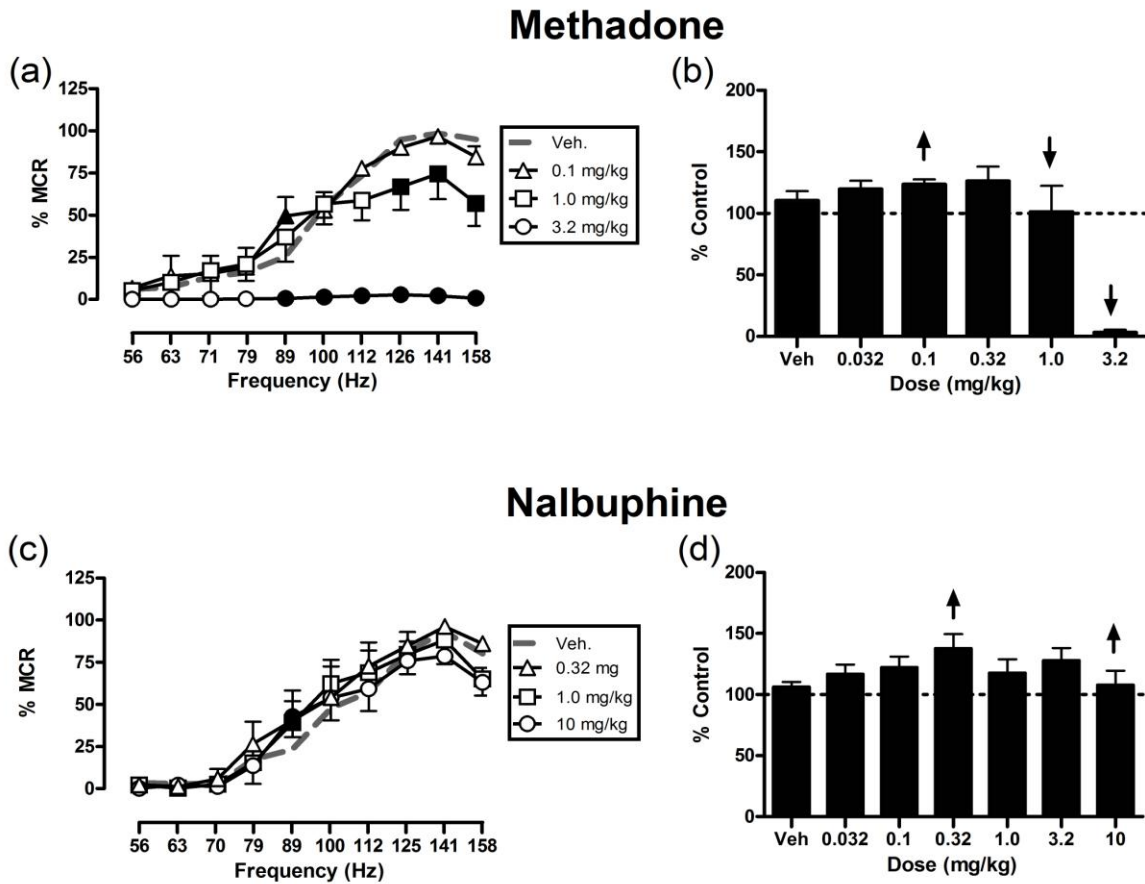


Figure 3.3. Effects of the high-efficacy m agonist methadone (a, b) and the low-efficacy nalbuphine (c, d) on ICSS in separate groups of opioid-naive rats. ANOVA results were as follows: methadone: significant main effect of frequency [F(9,45)=33.8; P<0.001], significant main effect of dose [F(5,25)=18.5; P<0.001], and significant dose x frequency interaction [F(45,225)=8.2; P<0.001]; nalbuphine: significant main effect of frequency [F(9,45)=30.0; P<0.001], no significant main effect of dose [F(6,30)=2.2; P=0.074], and significant dose x frequency interaction [F(54,270)=2.2; P<0.001]. For a description of axes and symbols, please refer to Fig. 3.1. ANOVA, analysis of variance; ICSS, intracranial self-stimulation; MCR, maximum control rate; Veh, vehicle.

Table 3.1. Control MCR and Total Stimulations obtained during experiments to determine the effects of mu-opioid receptor ligands.

	MCR \pm SE	Total Stimulations \pm SE
Mu ligands (Opioid experienced)	54.8 \pm 12.6	291.7 \pm 82.6
Methadone (naïve)	57.1 \pm 6.1	221.5 \pm 48.4
Nalbuphine (naïve)	57.3 \pm 10.6	207.8 \pm 91.6

CHAPTER FOUR

Abuse-related effects of mu opioid analgesics in an assay of intracranial self-stimulation in rats: modulation by chronic morphine exposure

(Behavioural Pharmacology; submitted)

4.1. Introduction

The purpose of the present study was to extend my previous research on determinants of mu opioid agonists on ICSS in rats (Altarifi and Negus, 2011; Altarifi *et al.*, 2012). Specifically, my previous studies identified two general phenomena. First, effects of mu agonists on ICSS in subjects with no prior history of opioid exposure were dependent on the efficacy of the opioid at mu receptors (with in vitro efficacy to stimulate GTP γ S binding as the metric of efficacy; Selley *et al.*, 1998). High- to intermediate-efficacy mu agonists such as methadone and morphine produced biphasic effects that included both increases in low ICSS rates maintained by low brain stimulation frequencies and decreases in high ICSS rates maintained by high brain stimulation frequencies. Conversely, lower efficacy mu agonists such as nalbuphine were less effective to produce both rate-increasing and rate-decreasing effects, and the opioid antagonist naltrexone did not alter ICSS at doses that blocked the effects of mu agonists (Chapter 3). Second, with morphine, chronic treatment produced tolerance to

rate-decreasing effects and enhanced expression of rate-increasing effects, and prevailing evidence suggests that this differential tolerance reflects greater desensitization by chronic morphine of mu receptors mediating rate-decreasing than rate-increasing effects (Altarifi *et al.*, 2012; Miller *et al.*, submitted).

The present study examined the interaction between these two phenomena by evaluating effects of chronic morphine exposure on changes in ICSS produced by four mu opioid receptor ligands that vary in efficacy at mu receptors: the high-efficacy mu agonist methadone, the intermediate efficacy mu agonist fentanyl, the low-efficacy mu agonist nalbuphine, and the mu antagonist naltrexone (Selley *et al.*, 1998). I

hypothesized that chronic morphine would produce the following efficacy-dependent effects: (1) tolerance to rate-decreasing effects for all agonists, with the potential for greater tolerance to lower efficacy agonists; (2) lesser or no tolerance to rate-increasing effects for all agonists, with the potential for greater tolerance to the lower efficacy agonists; and (3) dependence as indicated by the emergence of withdrawal-associated depression of ICSS by naltrexone and possibly also by nalbuphine.

4.2. Methods

4.2.1. Subjects

Subjects are similar to those described on section 2.2.1.

4.2.2. Assay of intracranial self-stimulation

Intracranial self-stimulation electrode implantation and behavioral procedure are similar to those described on section 2.2.2.

Testing: Once training and habituation to saline injections were completed, “pre-drug baseline” sessions were conducted over a period of 3 consecutive days to establish baseline ICSS performance before administration of any mu agonists. Each pre-drug baseline session consisted of 3 components as described in section 2.2.2. Rats were then distributed into 3 different groups. Each group received a different test drug (methadone 0.32-5.6 mg/kg; fentanyl 0.003-0.1 mg/kg; or nalbuphine 0.1-10 mg/kg). Testing in each group proceeded in three phases to evaluate test drug effects before chronic morphine (phase 1), during daily treatment with 3.2 mg/kg/day morphine (phase 2) and during treatment with 18 mg/kg/day morphine (phase 3). In addition to being tested with their designated test mu agonist during each phase, all rats were also tested with 0.1 mg/kg naltrexone after mu agonist testing during phase 3. Rats in the methadone and nalbuphine groups were also tested again 3 weeks after termination of repeated morphine (phase 4). Table 4.1 summarizes the sequence of treatments in all groups.

The first phase started immediately after the third pre-drug baseline session and lasted for 15-20 days. Daily ICSS sessions in this and all subsequent phases consisted of (a) three consecutive daily-baseline components, (b) a 30 min time out, with administration of saline or drug at the beginning of the time out, and (c) two more test components. Thus, ICSS was assessed twice each day: once during the daily-baseline components before that day’s injection of saline or drug (and approximately 23 hr after

the previous day's injection), and once during the test components that began 30 min after that day's injection. After the last component, subjects were removed from the test chamber and returned to their home cages. Test sessions involving administration of active doses of the test drug were separated by at least three days in the fentanyl group, which was the first group studied. A modification in experimental design was introduced for the later methadone and nalbuphine groups (see below for rationale), and in these groups, test sessions with active doses were separated by at least two days. For all groups, saline was administered instead of test drug on intervening days. ICSS sessions were sometimes omitted on weekends.

The second phase started with a 7-day maintenance period, during which 3.2 mg/kg/day morphine was administered during the time out of each daily ICSS session. On day 8, test sessions were resumed, and test drug effects were redetermined using the same dose order and intervals as the first phase. In addition, the fentanyl group was also tested with an additional higher dose in phase two after testing with the original doses was completed. On days that subjects did not receive test drug, they received 3.2 mg/kg morphine. This morphine injection was usually administered during the time out of an ICSS session as described above; however, ICSS sessions were occasionally omitted during the weekends, and on these days, the morphine injection was administered without ICSS. As in phase 1, test sessions for fentanyl were separated by at least three days, so that each test session was preceded by at least two days of treatment with the chronic morphine dose. In the methadone and nalbuphine groups, an alternative design was implemented to minimize protracted opioid withdrawal on days when saline or low test-drug doses were examined. Thus, when subjects were

tested with saline vehicle or with low doses of methadone (0.32 mg/kg) or nalbuphine (0.1-0.32 mg/kg), they also received a supplemental dose of 3.2 mg/kg morphine after the final component on that day, before returning to their home cages. Supplemental doses were not administered after higher doses of methadone or nalbuphine to minimize the potential for opioid overdose. Phase two lasted 20-25 days.

The third phase began with a gradual increase in the morphine dose administered during the time out of consecutive daily ICSS sessions. Typically, subjects received 5.6 mg/kg/day morphine for 2 days, followed by 10 mg/kg/day morphine for 2-4 days, followed by the terminal dose of 18 mg/kg/day for the remainder of the third phase. The rate of dose escalation was individually determined in each rat to assure expression of ICSS at a given dose before proceeding to a higher dose. Once the terminal dose of 18 mg/kg/day morphine was achieved, it was maintained for seven days. Subsequently, test sessions were resumed, and test drug effects were redetermined using the same dose order and intervals as in the second phase. In addition, the methadone group was also tested with an additional higher dose in phase 3 after testing with the original doses was completed. Also, all subjects were tested with 0.1 mg/kg naltrexone (NLTX) after testing with the designated mu agonist was completed. This naltrexone dose was selected as a dose that did not alter ICSS in non-dependent rats but that blocked methadone-induced facilitation of ICSS in a previous study (Altarifi *et al.*, 2012). Subjects in this phase received 18 mg/kg/day morphine on the days that they did not receive test drug, and 18 mg/kg morphine was also administered at the end of test sessions during which saline vehicle or low methadone

(0.32-1.0 mg/kg), or nalbuphine (0.1-3.2 mg/kg) doses were tested. Phase three lasted between 20-25 days.

At the end of the third phase, daily morphine injections were terminated, but daily ICSS sessions continued for at least three days. No further experiments were conducted in the fentanyl group. For the methadone and nalbuphine groups, ICSS sessions and drug treatments were suspended for two weeks. Training was then resumed for three days, after which an extra phase (phase four) was conducted in these subjects identical to phase 1.

4.2.3. Drugs

Morphine sulfate, methadone HCl, naltrexone HCl and fentanyl HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Nalbuphine HCl was provided by Dr. Kenner Rice (Chemical Biology Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). All drugs were dissolved in saline and delivered subcutaneously in a volume of 1 ml/kg body weight.

4.2.4. Data Analysis

The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial in each rat were converted to Percent Maximum Control Rate (%MCR) for that rat. The maximum control rate was determined for each rat during the pre-drug baseline sessions at the beginning of the experiment. The first component from these sessions

(and from all other sessions) was considered to be an acclimation component, and data were discarded. The maximum control rate was defined as the mean of the maximal rates observed during any frequency trial of the second and third components of the three pre-drug baseline sessions (six total pre-drug baseline components).

Subsequently, %MCR for each trial was calculated as (Reinforcement Rate During a Frequency Trial ÷ Maximum Control Rate) × 100. Graphs show mean frequency-rate curves, with brain stimulation frequency on the abscissa, and ICSS rate expressed as %MCR on the ordinate.

Frequency-rate curves from test sessions during each phase of the study were submitted for analysis. As noted above, these frequency-rate curves were assessed twice on each test day: once during daily baseline components before that day's injection (and approximately 23 hr after the previous day's injection), and again during test components that began 30 min after that day's injection. Daily baseline data and test data from test sessions were analyzed separately. The daily baseline data provided information on changes in baseline ICSS produced by the chronic treatment (saline, 3.2 mg/kg/day morphine, or 18 mg/kg/day morphine). More specifically, because daily baseline components were conducted approximately 23 hr after the most recent injection of the chronic treatment, they provided data on changes in ICSS produced by 23 hr withdrawal from that treatment. Because rats in all three test drug groups received the same progression of chronic morphine treatments during sequential phases of the study, daily baseline data from test sessions within each phase were averaged across all rats to yield mean baseline ICSS data during chronic treatment with saline, 3.2 mg/kg/day morphine and 18/mg/kg/day morphine. These mean baseline

data during each phase were compared to the pre-drug baseline data using two-way ANOVA, with phase of chronic treatment as one factor and ICSS frequency as the other factor. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$. To facilitate within-subject data analysis, data were included only for those rats that completed all three phases of chronic morphine treatment (N=15, 5 from each group).

Test data from each test session were analyzed to assess dose effects of each test drug (methadone, fentanyl, nalbuphine) on ICSS frequency-rate curves during each phase of chronic morphine treatment. Within each phase, ICSS test data for a given dose were averaged across rats and compared by two-way ANOVA, with drug dose as one factor and ICSS frequency as the other factor. A similar approach was used to compare effects of saline and 0.1 mg/kg naltrexone treatment during phase 3. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$. Data were included for all rats that completed a given phase.

To provide an additional summary measure of baseline and test ICSS performance, the total number of stimulations per component was calculated as the average of the total stimulations delivered across all 10 frequency trials of each component. Baseline and test data were expressed as a percentage of the total stimulations per component earned during the “pre-drug baseline” components (% Control). Thus, % Control was calculated as (Mean Total Stimulations During Daily Baseline or Test Components ÷ Mean Total Stimulations During Pre-Drug Baseline Components) × 100.

4.3. Results

Baseline ICSS before and during chronic morphine. During pre-drug baseline sessions, the average maximum control rate (MCR) for all rats in the study was 57.2 ± 9.8 stimulations per trial, and the average control number of total stimulations delivered across all frequencies was 286.7 ± 70.7 . Table 4.2 shows the MCR and control number of total stimulations for each group of rats. Figure 4.1 shows mean frequency-rate ICSS curves before treatment and during phases 1-3 of chronic morphine treatment. During baseline (i.e. before any drug administration), little responding was maintained by the lower frequencies of stimulation (56-79 Hz), and ICSS increased at the intermediate and higher frequencies (89-158 Hz). Chronic treatment with vehicle (phase 1) and 3.2 mg/kg morphine (phase 2) did not significantly alter ICSS. During phase 3, treatment with repeated 18 mg/kg/day morphine produced a decrease in ICSS relative to the pre-drug baseline (significant at 100-126 Hz). ICSS partially recovered within three days after termination of chronic morphine, although ICSS rates were still significantly depressed at one frequency (112 Hz).

Effects of methadone on ICSS before and during chronic morphine. Figure 4.2 shows the effect of methadone on ICSS during phases 1-3. During phase 1, methadone failed to facilitate ICSS at any brain stimulation frequency. Rather, methadone dose-dependently decreased ICSS maintained by high frequencies of brain stimulation, with significant depression at 126-158 Hz after 1.0 mg/kg methadone and at 100-158 Hz after 3.2 mg/kg methadone (figure 4.2 a,b). Repeated treatment with 3.2 mg/kg/day morphine during phase 2 reduced expression of methadone's rate-decreasing effects and increased expression of its rate-increasing effects. Thus, 1.0

mg/kg methadone no longer depressed ICSS at any frequency of brain stimulation, but rather produced facilitation of ICSS at intermediate frequencies (71-100 Hz). Similarly, 3.2 mg/kg depressed ICSS across a narrower range of high frequencies during phase 2 (141-158 Hz), and it increased ICSS at intermediate frequencies (71-79 Hz). One of eight subjects died before completion of this phase, so only seven rats completed testing with all methadone doses in phase 2 and advanced to phase 3. During phase 3, 1.0 and 3.2 mg/kg methadone produced exclusive rate-increasing effects at the intermediate frequencies 89-100 Hz and 63-89 Hz, respectively. A higher dose of 5.6 mg/kg methadone was introduced during this phase, and it produced biphasic effects similar to the effects of 3.2 mg/kg during phase 2 (figure 4.2 e,f). Two subjects died during high-dose morphine treatment in phase 3, so only 5 rats completed testing with all methadone doses in this phase.

Effects of fentanyl on ICSS before and during chronic morphine. Figure 4.3 shows the effect of fentanyl on ICSS during phases 1-3. During phase 1, a low dose of 0.003 mg/kg fentanyl facilitated ICSS at one frequency (89 Hz), but only rate-decreasing effects were produced by higher fentanyl doses of 0.01 mg/kg (158 Hz) and 0.03 mg/kg (100-158) (figure 4.3 a,b). A lower fentanyl dose of 0.001 mg/kg was also tested, and it did not produce any change in ICSS (data not shown). During phase 2, 0.003 mg/kg fentanyl did not produce any significant change in ICSS compared to vehicle. However, effects of 0.01 and 0.03 mg/kg fentanyl changed from exclusive depression of ICSS at high frequencies during phase 1 to exclusive facilitation of ICSS at intermediate frequencies during phase 2. Thus, fentanyl at 0.01 and 0.03 mg/kg produced exclusive facilitation of ICSS at 89-100 Hz and 71-100 Hz, respectively (figure 4.3 c,d). A higher

fentanyl dose of 0.1 mg/kg was also introduced during phase 2, and this dose nearly eliminated responding and significantly reduced ICSS at the highest five frequencies (100-158 Hz). One of six rats died during phase 2, so only five rats completed testing with all fentanyl doses and advanced to phase 3. During the third phase, neither 0.003 nor 0.01 mg/kg morphine altered ICSS at any frequency. However, 0.03 mg/kg fentanyl still facilitated ICSS at intermediate frequencies (71-100 Hz), and 0.1 mg/kg fentanyl still depressed ICSS at high frequencies (112-158 Hz) (figure 3 e,f).

Effects of nalbuphine on ICSS before and during chronic morphine. Figure 4.4 shows the effects of nalbuphine on ICSS during phases 1-3. During phase 1, nalbuphine primarily facilitated ICSS, although these effects were not monotonically related to dose. Thus, exclusive facilitation of ICSS was produced by nalbuphine doses of 0.1 (79 Hz), 0.32 (71-89 Hz) and 3.2 mg/kg (71-89 Hz), and the highest dose of 10 mg/kg produced biphasic effects (facilitation at 63-79 Hz and depression at 158 Hz). Conversely, 1.0 mg/kg nalbuphine (the first dose tested) did not facilitate ICSS and significantly depressed ICSS at the highest frequency (158 Hz). Repeated treatment with 3.2 mg/kg/day morphine during phase 2 eliminated expression of nalbuphine's rate-decreasing effects and enhanced the dose-dependence and magnitude of nalbuphine's rate-increasing effects. For example, 10 mg/kg nalbuphine facilitated ICSS at frequencies of 63-100 Hz and did not depress ICSS at any frequency (figure 4.4 c,d). During phase 3, nalbuphine produced similar effects consisting of exclusive ICSS facilitation across all nalbuphine doses. In addition, diarrhea was observed in all subjects after administration of 0.32-3.2 mg/kg nalbuphine (data not shown). One of six

rats died during phase 3, so only five rats completed testing with all nalbuphine doses in this phase.

Effect of naltrexone on ICSS during chronic 18 mg/kg/day morphine. At the end of phase 3 and before the termination of the daily morphine treatment, 0.1 mg/kg naltrexone was tested and results are shown in figure 4.5. Rate depression was the predominant effect of naltrexone compared to vehicle, and it depressed ICSS at frequencies of 112 and 126 Hz. Diarrhea was observed in all subjects after administration of this dose of naltrexone.

Effect of methadone and nalbuphine after 2 weeks of morphine abstinence. The effects of methadone (N=5) and nalbuphine (N=5) were redetermined beginning after 3 weeks of morphine abstinence. As noted above, daily baseline ICSS recovered toward pre-drug baseline levels within three days after termination of morphine treatment (Figure 4.1), and daily baseline ICSS persisted at pre-drug baseline levels throughout testing during abstinence. For example, for the 10 rats that completed abstinence testing, the pre-drug control \pm SEM number of stimulations per component was 297.8 ± 83.6 , and the mean number of stimulations per component during abstinence testing was 293.0 ± 163.0 . Figure 4.6 shows that methadone and nalbuphine effects during abstinence testing were generally similar to their effects during phase 2 testing. Thus, 0.32 mg/kg methadone did not significantly alter ICSS, 1.0 mg/kg methadone facilitated ICSS at intermediate frequencies (63-100 Hz), and 3.2 mg/kg methadone tended to produce biphasic effects, with facilitation at 71-79 Hz and a non-significant decrease in mean ICSS rates at high frequencies (126-158 Hz) (figure 4.6 a,b). Similarly, nalbuphine produced a dose-dependent facilitation of ICSS, although

effects of the lowest dose of 0.1 mg/kg nalbuphine did not achieve statistical significance (figure 4.6 c,d). In addition, diarrhea was not detected after any nalbuphine dose during this phase (data not shown).

4.4. Summary

This study examined the impact of graded morphine exposure on changes in ICSS produced by agonists with high efficacy (methadone), intermediate efficacy (fentanyl) or low efficacy (nalbuphine) at mu opioid receptors. There were three main findings. First, in agreement with previous results (Altarifi *et al.*, 2012) the higher efficacy mu agonists methadone and fentanyl produced primarily rate-decreasing effects in opioid-naïve subjects, whereas the low-efficacy mu agonist nalbuphine produced primarily rate-increasing effects that did not vary systematically as a function of dose. Second, repeated morphine produced cross tolerance to the rate-decreasing effects and enhanced expression of the rate-increasing effects of all three mu agonists. Lastly, the daily morphine dosing regimen used here produced withdrawal-associated decreases in baseline ICSS determined approximately 23 hr after morphine. Repeated morphine also enhanced rate-decreasing effects of the antagonist naltrexone. However, this evidence of opioid dependence and withdrawal was not sufficient to account for enhanced expression of mu agonist-induced rate-increasing effects. Taken together, these results provide further evidence to suggest that repeated opioid exposure increases the degree to which mu agonists produce abuse-related facilitation of ICSS.

Figure 4.1

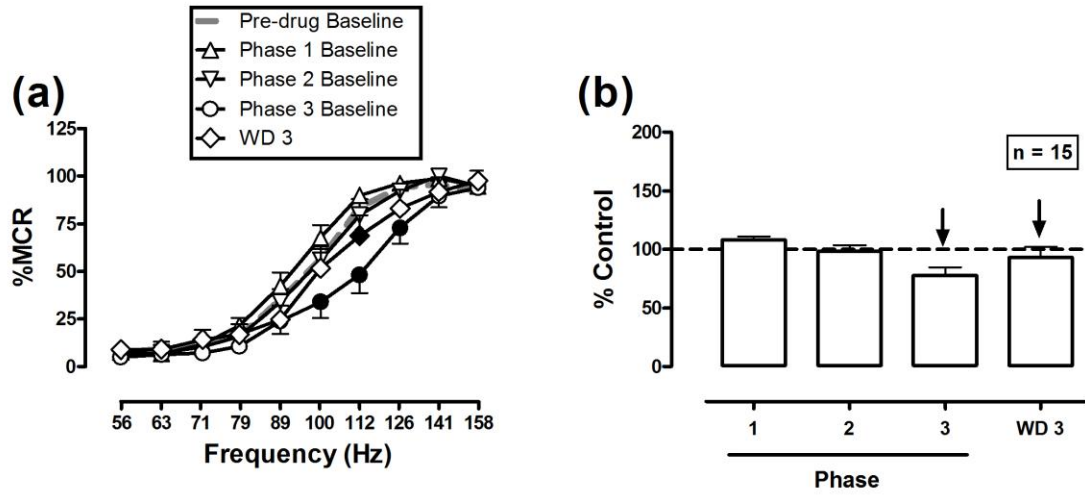
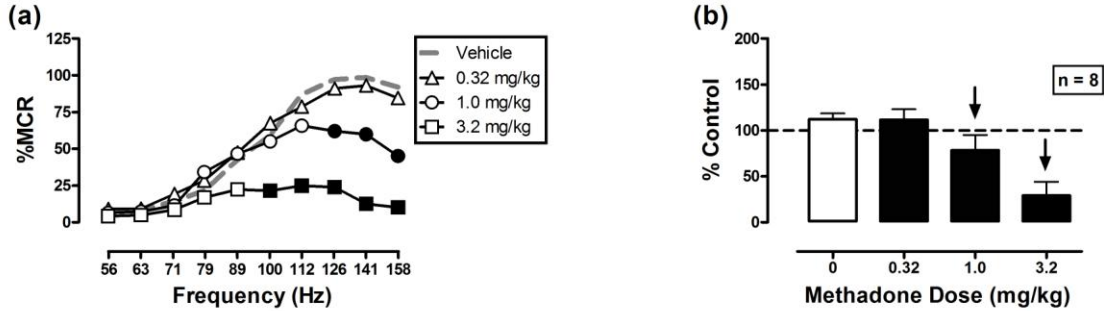


Figure 4.1. ICSS performance before, during and after chronic morphine

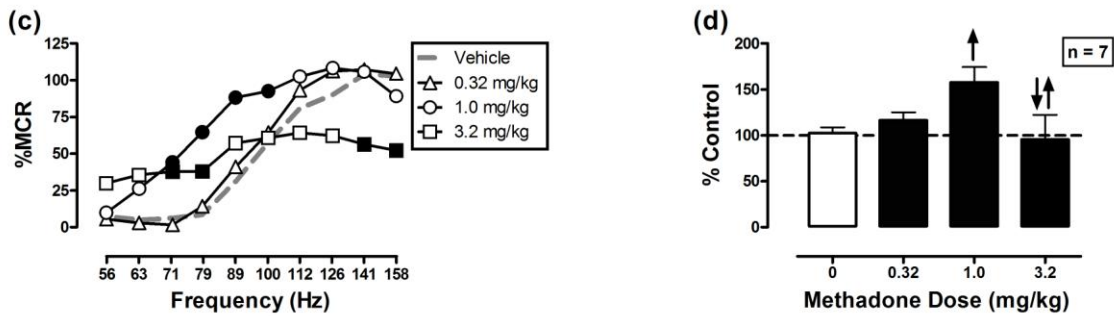
treatment. ICSS curves were analyzed during pre-drug baseline sessions (grey dashed line), daily baseline components from test sessions during phases 1-3, and day 3 after termination of chronic morphine treatment (WD3: Withdrawal Day 3) for subjects that finished all three phases. The left panel shows ICSS frequency-rate curves. Horizontal axes: frequency of electrical brain stimulation in hertz (log scale). Vertical axes: ICSS rate expressed as percent maximum control rate (%MCR). Filled symbols indicate frequencies at which ICSS rates were lower than those observed during the pre-drug baseline components, as determined by the Holm-Sidak post-hoc test following a significant two-way ANOVA. Summary data in the right panel show the total number of stimulations per test component expressed as a percentage of total pre-drug baseline control stimulations. Horizontal axes: phase of the treatment. Vertical axes: percent control stimulations per test component. Upward and/or downward arrows indicate the presence and valence of significant differences from pre-drug baseline as determined by analyses of frequency-rate data in the left panel. Thus, upward arrows indicate significant facilitation of ICSS at ≥ 1 frequency of the frequency-rate curve, whereas downward arrows indicate significant depression of ICSS at ≥ 1 frequency of the frequency-rate curve. ANOVA results were as follows: Significant main effect of frequency [$F(9,126)=158.1$; $P<0.001$], significant main effect of phase [$F(3,42)=11.0$; $P<0.001$], and significant phase X frequency interaction [$F(27,378)=4.9$; $P<0.001$]. All points show mean \pm SEM for 15 rats.

Figure 4.2

Chronic Vehicle



Chronic 3.2 mg/kg/day morphine



Chronic 18 mg/kg/day morphine

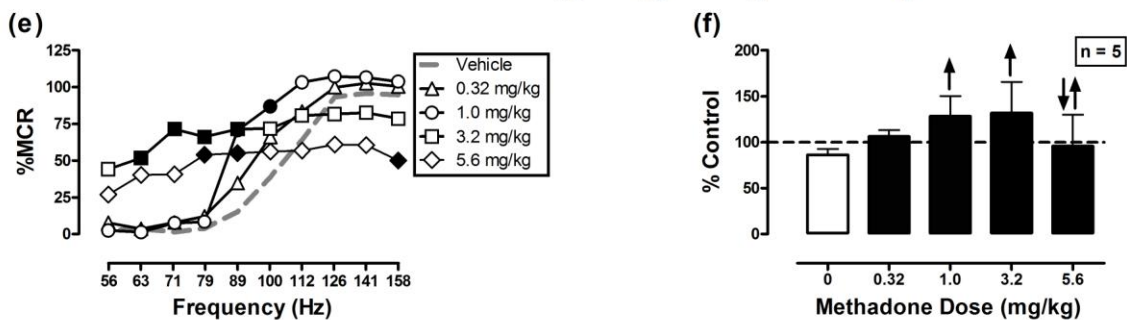
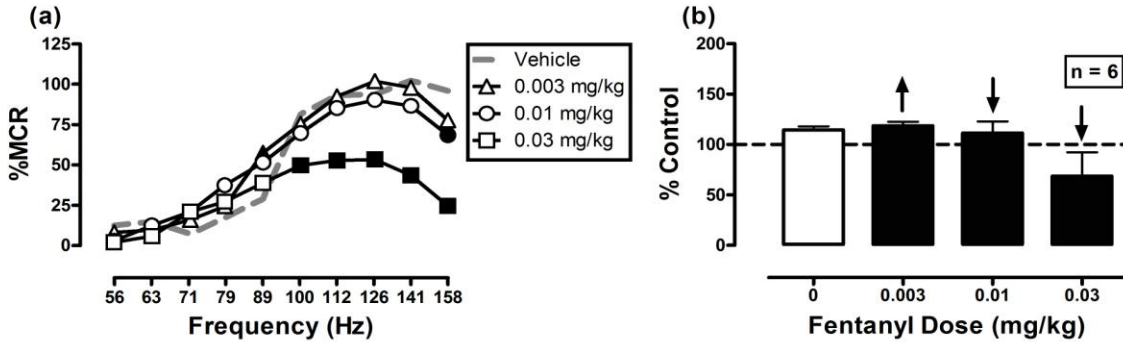


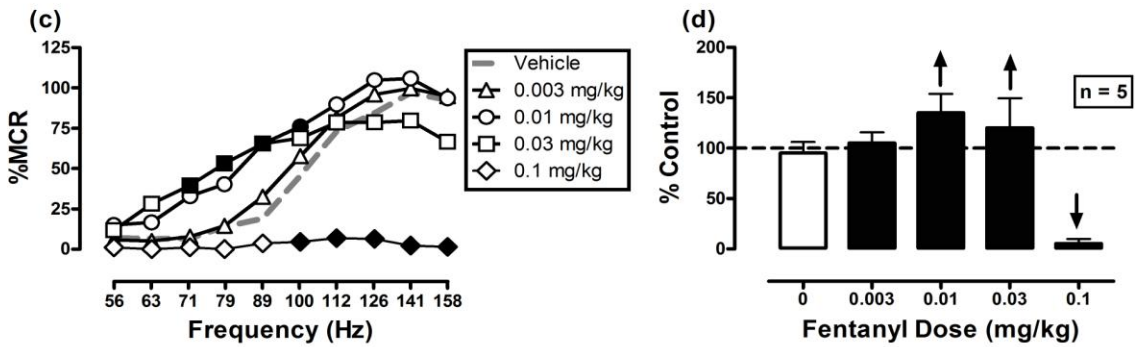
Figure 4.2. Effects of methadone on ICSS before and during chronic morphine treatment. Methadone doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). Left panels show full frequency-rate curves. Left abscissae: frequency of electrical brain stimulation in hertz (log scale). Left ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled symbols indicate frequencies at which ICSS rates after methadone were different than those observed after vehicle, as determined by the Holm-Sidak post-hoc test following a significant two-way ANOVA. Summary data in the right panels show the total number of stimulations per test component expressed as a percentage of total pre-drug baseline control stimulations. Abscissae: dose of methadone in mg/kg. Ordinates: percent control stimulations per test component. Upward and/or downward arrows indicate the presence and valence of significant differences from vehicle treatment as determined by analyses of frequency-rate data in the left panels. All points show mean \pm SEM for 5-8 rats. For description of axes and symbols, please refer to figure 1. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,63)=31.4$; $P<0.001$], significant main effect of dose [$F(3,21)=9.9$; $P<0.001$], and significant dose X frequency interaction [$F(27,189)=9.1$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,54)=67.2$; $P<0.001$], no significant main effect of dose [$F(3,18)=2.6$; $P=0.086$], and significant dose X frequency interaction [$F(27,162)=11.2$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=91.5$; $P<0.001$], no significant main effect of dose [$F(4,16)=1.2$; $P=0.365$], and significant dose X frequency interaction [$F(36,144)=10.4$; $P<0.001$].

Figure 4.3

Chronic Vehicle



Chronic 3.2 mg/kg/day morphine



Chronic 18 mg/kg/day morphine

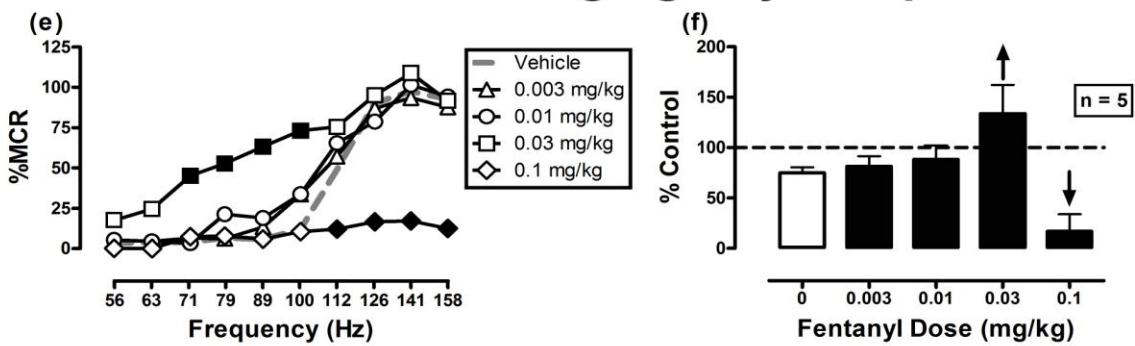
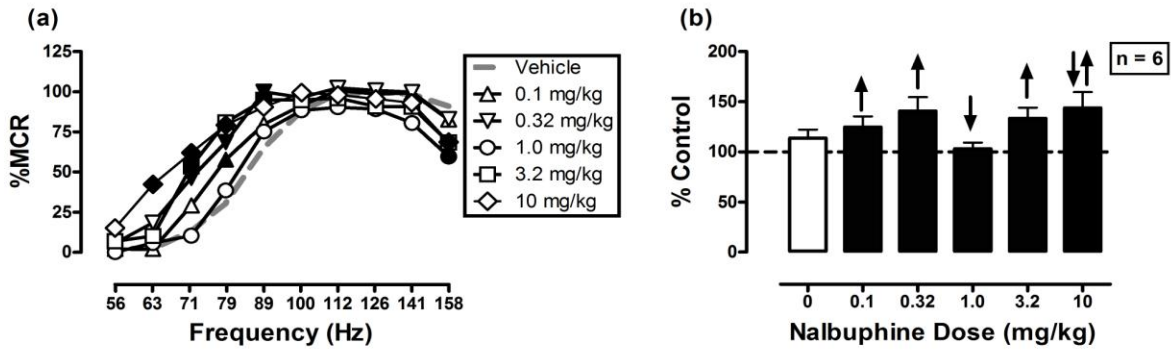


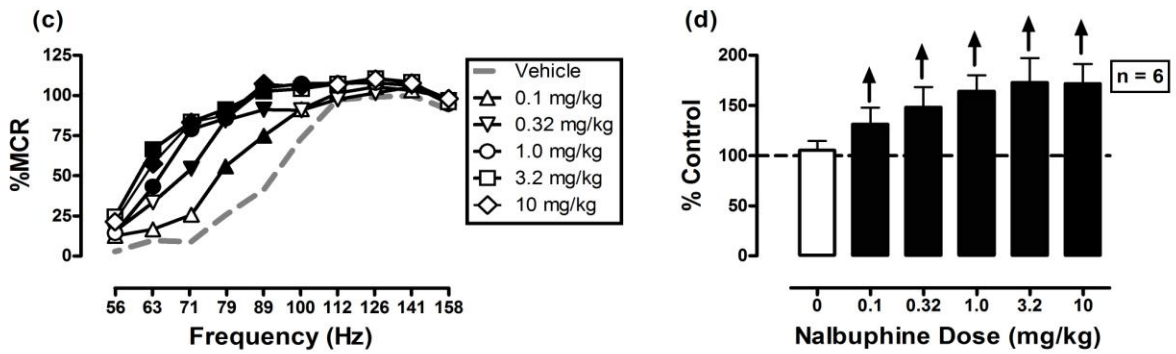
Figure 4.3. Effects of fentanyl on ICSS before and during chronic morphine treatment. Fentanyl doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). All points show mean \pm SEM for 5-6 rats. For description of axes and symbols, please refer to figure 2. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,45)=93.2$; $P<0.001$], significant main effect of dose [$F(3,15)=4.3$; $P=0.022$], and significant dose X frequency interaction [$F(27,135)=5.6$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,45)=30.6$; $P<0.001$], significant main effect of dose [$F(4,20)=15.7$; $P<0.001$], and significant dose X frequency interaction [$F(36,180)=10.0$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=37.7$; $P<0.001$], significant main effect of dose [$F(4,16)=12.1$; $P<0.001$], and significant dose X frequency interaction [$F(36,144)=5.3$; $P<0.001$].

Figure 4.4

Chronic Vehicle



Chronic 3.2 mg/kg/day morphine



Chronic 18 mg/kg/day morphine

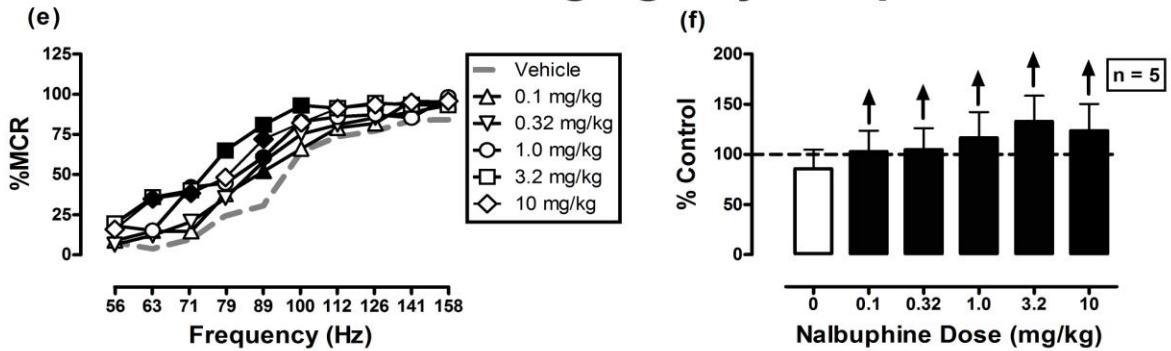


Figure 4.4. Effects of nalbuphine on ICSS before and during chronic morphine treatment. Nalbuphine doses (or vehicle) were administered during treatment with repeated vehicle (phase 1; a,b), repeated 3.2 mg/kg/day morphine (phase 2; c,d), and repeated 18 mg/kg/day morphine (phase 3; e,f). All points show mean \pm SEM for 5-6 rats. For description of axes and symbols, please refer to figure 2. ANOVA results were as follows: Chronic vehicle: Significant main effect of frequency [$F(9,45)=63.0$; $P<0.001$], significant main effect of dose [$F(5,25)=6.6$; $P<0.001$], and significant dose X frequency interaction [$F(45,225)=3.0$; $P<0.001$]. Repeated 3.2 mg/kg/day morphine: Significant main effect of frequency [$F(9,45)=18.9$; $P<0.001$], significant main effect of dose [$F(5,25)=14.9$; $P<0.001$], and significant dose X frequency interaction [$F(45,225)=2.9$; $P<0.001$]. Repeated 18 mg/kg/day morphine: Significant main effect of frequency [$F(9,36)=13.2$; $P<0.001$], significant main effect of dose [$F(5,20)=9.0$; $P<0.001$], but no significant dose X frequency interaction [$F(45,180)=1.0$; $P=0.532$].

Figure 4.5

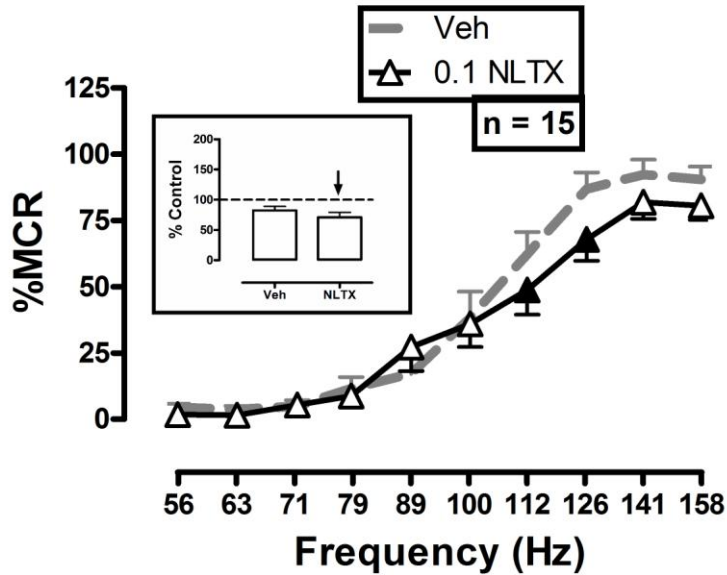


Figure 4.5. Effects of 0.1 mg/kg naltrexone (NLTX) on ICSS during chronic 18 mg/kg/day morphine treatment (phase 3). All points show mean \pm SEM for 15 rats from all groups. For description of axes and symbols, please refer to figure 2. There was significant main effect of frequency [$F(9,126)=74.0$; $P<0.001$], significant main effect of treatment [$F(1,14)=6.7$; $P=0.021$], and significant dose X frequency interaction [$F(9,126)=3.7$; $P<0.001$].

Figure 4.6

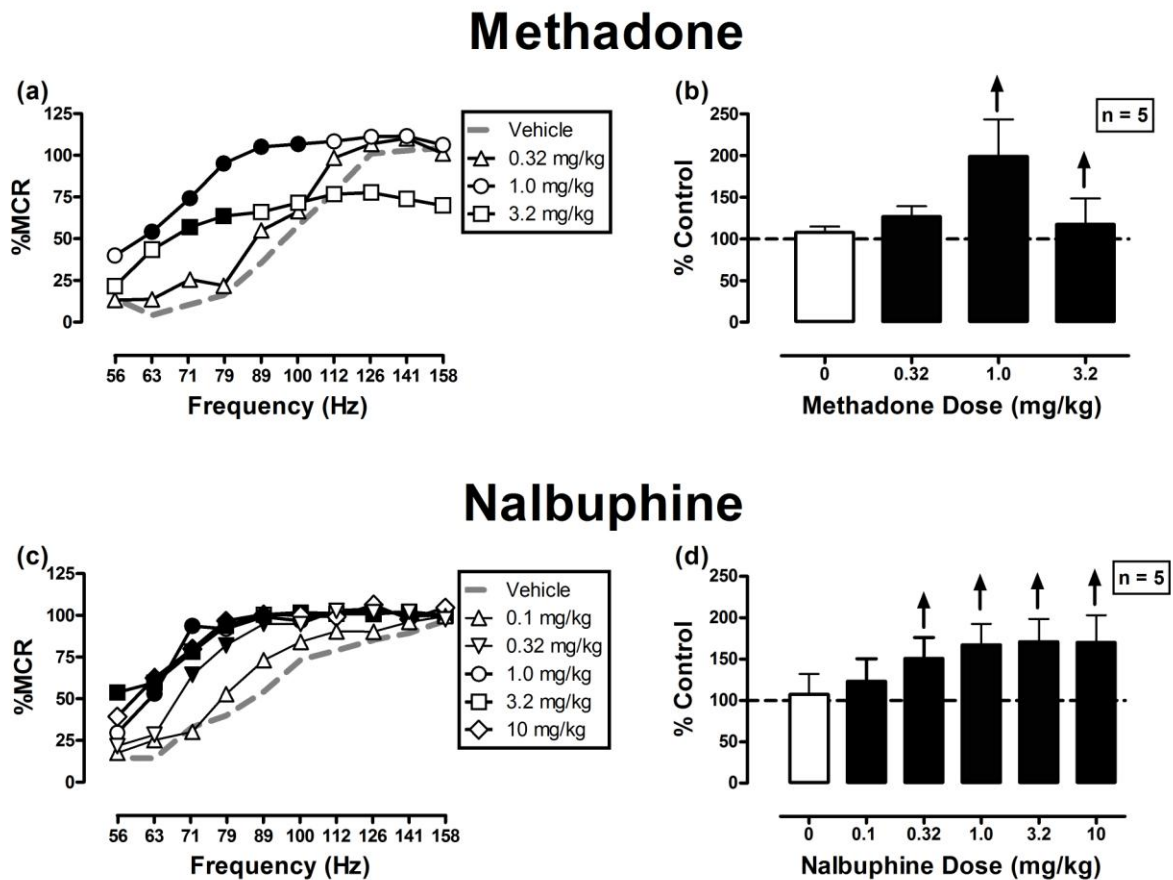


Figure 4.6. Effects of methadone and nalbuphine on ICSS after termination of repeated morphine treatment. Methadone (a,b) and nalbuphine (c,d) were tested after 3 weeks of morphine abstinence. All points show mean \pm SEM for 5 rats. For description of axes and symbols, please refer to figure 2. ANOVA results were as follows: Methadone: Significant main effect of frequency [$F(9,36)=22.6$; $P<0.001$], no significant main effect of dose [$F(3,12)=2.6$; $P=0.097$], and significant dose X frequency interaction [$F(27,108)=5.7$; $P<0.001$]. Nalbuphine: Significant main effect of frequency [$F(9,36)=7.8$; $P<0.001$], significant main effect of dose [$F(5,20)=11.3$; $P<0.001$], and significant dose X frequency interaction [$F(45,180)=2.0$; $P<0.001$].

Table 4.1. Summary table showing the experimental design, drug doses, and number of subjects used in each group

Group	Test Drug	Variable	Phase I	Phase II	Phase III	Phase IV
1	Methadone (n = 5-8)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	Vehicle
		Doses (mg/kg)	0.032-3.2	0.032-3.2	0.032-5.6	0.032-3.2
2	Fentanyl (n = 5-6)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	N/A ^a
		Doses (mg/kg)	0.001-0.03	0.001-0.1	0.001-0.1	N/A ^a
3	Nalbuphine (n = 5-6)	Chronic treatment	Vehicle	3.2 mg/kg/day morphine	18 mg/kg/day morphine	Vehicle
		Doses	0.1-10	0.1-10	0.1-10	0.1-10

^a N/A=not applicable. Subjects were euthanized at the end of phase III

Table 4.2. Maximum control rates (MCR) and total stimulations obtained during pre-drug Baseline

Group (Drug)	MCR (\pm SEM)	Control Total Stimulations (\pm SEM)
1 (Methadone)	56.5 (\pm 6.8)	282.5 (\pm 61.9)
2 (Fentanyl)	55.8 (\pm 11.1)	263.5 (\pm 52.6)
3 (Nalbuphine)	59.7 (\pm 12.7)	315.5 (\pm 96.0)

CHAPTER FIVE

Effects of mu opioid receptor agonists in assays of acute pain-stimulated and pain-depressed behavior in rats

5.1. Introduction

Pain-related behaviors can be assigned to two general categories that we have called “pain-stimulated behaviors” and “pain-depressed behaviors” (Negus *et al.*, 2010). Most assays that are used to measure pain in laboratory animals fall under pain-stimulated behaviors, such as tail-withdrawal, paw withdrawal, and stretching. Although these types of assays helped in identifying pain neurobiology, identifying possible targets for future drug analgesics, and studying the pharmacology of current analgesics, they failed to generate more efficient and safer analgesics than those currently used in pain medicine, such as opioids and NSAIDs. One reason behind this discrepancy is that animal ‘pain’ models do not simulate multidimensional clinical pain conditions (Mao, 2012). On the other hand, pain-depressed behaviors may be more clinically relevant, but they have received little attention in preclinical research. Thus, preclinical assays of pain-depressed behaviors, such as feeding and locomotion, may be a better target to enhance pain medicine.

It has been shown previously that intraperitoneal injection of dilute lactic acid produced depression in ICSS and pretreatment with morphine was able to block the acid effect (Pereira Do Carmo *et al.*, 2009). Depression of ICSS can be classified as an example of pain-depressed behaviors, and the effect of morphine may indicate the sensitivity of this assay to clinically used analgesics. In the current study, lactic acid was used as the noxious stimulus because hydrogen ions can directly activate acid-sensitive ion channels which have a particular relevance in the development and maintenance of inflammatory pain (Karczewski *et al.*, 2010), and we wanted to expand these findings by testing the pharmacology of mu opioid agonists with and without noxious stimulus in ICSS in comparison to an assay of acid-stimulated stretching. We tested a variety of clinically used opioid agonists that have different efficacy at the mu receptor. Also, selected agonists were tested after noxious stimulus intensity manipulation.

5.2. Methods

5.2.1. Subjects

Subjects are similar to those described on section 2.2.1.

5.2.2. Assay of intracranial self-stimulation

Intracranial self-stimulation electrode implantation and behavioral procedure are similar to those described on section 2.2.2.

Testing: ICSS testing was conducted in two phases. First, the effects of methadone (0.032-1.0 mg/kg), fentanyl (0.0032-0.032 mg/kg), morphine (0.1-3.2

mg/kg), hydrocodone (0.1-3.2 mg/kg), buprenorphine (0.001-0.032 mg/kg) and nalbuphine (0.1-1.0 mg/kg) were examined as pretreatments to 1.8% lactic acid or acid vehicle (sterile water). Each drug was tested in a separate group of 5-6 rats that were opioid-naïve at the start of the study. ICSS test sessions consisted of 5 sequential components with a 30 min time out between the first three and last two components. The first component of each test session was considered to be an acclimation component, and data 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). Following these baseline components and during the time out, test drug or its vehicle was administered subcutaneously as a 30-minute pretreatment to 1.8% lactic acid or its vehicle (IP in a volume of 1.0 ml/kg). Two sequential test components were conducted immediately after the second injection. Test sessions were conducted on Tuesdays and Fridays, with one test day each week devoted to evaluation of one dose of one opioid as a pretreatment to lactic acid, and the other day devoted to evaluation of the same dose of the same opioid as a pretreatment to acid vehicle. For all drugs, doses were delivered in a mixed order across rats. Three-component training sessions were conducted during other weekdays.

The second phase of the study was designed to compare effects of methadone and nalbuphine on depression of ICSS produced by a higher intensity noxious stimulus. First, 13 naïve rats were treated at weekly intervals with subcutaneous saline as a 30-minute pretreatment to vehicle, 0.56%, 1.8%, or 5.6% lactic acid (in order of testing; IP in a volume of 1.0 ml/kg). Test sessions began with three baseline components followed first by delivery of injections during a 30 min time out and then by two

consecutive test components. Subsequently, the rats were divided into two groups, and the effect of 5.6% lactic acid on ICSS were redetermined after pretreatment with methadone (0.1 and 1.0 mg/kg; N=6) or nalbuphine (1.0 or 10 mg/kg; N=7). For each drug, the lowest dose was the dose that produced peak antinociception against 1.8% lactic acid in the first phase of the study, and this was the first dose tested. Rats were then tested a week later with a 10-fold higher dose of each drug.

Data Analysis. The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial were converted to Percent Maximum Control Rate (%MCR) for that rat on that day. The maximum control rate was determined during control components of each test session and was defined as the mean of the maximal rates observed in any frequency trial during the second and third control components. Thus, %MCR for each trial was calculated as (Reinforcement Rate During a Frequency Trial ÷ Maximum Control Rate) × 100. Normalized data from the frequency trials of each pair of consecutive test components were then averaged across rats for display and for statistical analysis using two-way ANOVA, with drug dose or time as one factor and ICSS frequency as the other factor. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$.

To provide an additional summary of ICSS performance, the total number of stimulations obtained at all frequencies was summed for each test component and averaged across the two test components of each experimental session in each rat. Data for total stimulations per component were then expressed as a percentage of the baseline number of stimulations per component in each rat and averaged across rats.

These data were also used to quantify blockade of acid-induced depression of ICSS. Specifically, "percent acid blockade" was quantified using the equation $[(\text{test-acid})/(\text{baseline-acid})]*100$, where "test" was the total number of ICSS stimulations after treatment with drug + acid, "acid" was the total number of stimulations after acid alone, and "baseline" was the total number of stimulations in the absence drug or acid. For all drugs producing greater than 50% acid blockade, linear regression was used to calculate an ED50 and 95% confidence limits, with ED50 defined as the effective dose producing 50% acid blockade. For drugs that produced inverted U-shaped dose-effect curves (methadone, fentanyl, morphine), the ED50 value was determined from the ascending limb of the dose-effect curve. ED50 values were considered to be significantly different if 95% confidence limits did not overlap.

5.2.3. Assay of lactic acid-stimulated stretching

Behavioral Procedure. Test sessions were conducted once per week. Test drugs were administered subcutaneously 30 minutes prior to treatment with 1.8% lactic acid (IP in a volume of 1.0 ml/kg). Immediately after acid injection, rats were placed into acrylic test chambers (31.0 X 20.1 X 20.0 cm) for 30-minute observation periods. A stretch was operationally defined as a contraction of the abdomen followed by extension of the hind limbs, and the number of stretches during the observation period was counted. Effects of methadone (0.1-1.0 mg/kg), fentanyl (0.0032-0.032 mg/kg), morphine (0.1-1.0 mg/kg), hydrocodone (0.1-1.0 mg/kg), buprenorphine (0.00032-0.01 mg/kg) and nalbuphine (0.1-1.0 mg/kg) were examined in separate groups of 5-8 rats. Drug doses were tested in a mixed order across rats.

Similar to ICSS experiments, a separate group of naïve rats (n=7) was treated with subcutaneous saline as a 30-minute pretreatment to vehicle (water), 0.56%, 1.8%, or 5.6% lactic acid (in order of testing; IP in a volume of 1.0 ml/kg). After acid administration, rats were observed for 30 min, and the total number of stretches was counted as described previously.

Data Analysis. The primary dependent variable was the number of stretches counted during each observation period in each rat. To normalize these data, raw counts were converted to percent vehicle control using the equation $(\text{drug}/\text{vehicle}) \times 100$, where "drug" was the number of writhes observed after drug + acid, and "vehicle" was the number of writhes after drug vehicle + acid. These data were then averaged across rats. For all drugs producing greater than 50% reduction in stretching, linear regression was used to calculate an ED50 and 95% confidence limits, with ED50 defined as the effective dose producing 50% control writhing.

To evaluate significance during tests with different acid concentrations, data were averaged across rats, and one way-ANOVA was used for statistical analysis. A significant ANOVA was followed by a Dunnett post-hoc test with $P < 0.05$.

5.2.4. Drugs

Methadone HCl, fentanyl HCl, morphine sulfate, hydrocodone bitartrate and buprenorphine HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Nalbuphine HCl was provided by Dr. Kenner Rice (Chemical Biology Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). All opioids were dissolved in saline and

delivered SC in a volume of 1 ml/kg body weight. Lactic acid was purchased from Sigma Chemical Co. (St. Louis, MO), diluted in sterile water, and administered IP in a volume of 1 ml/kg body weight.

5.3. Results

Effects of Mu Agonists in the Assay of Acid-Stimulated Stretching

Across all 35 rats used for studies of acid-stimulated stretching, IP administration of 1.8% lactic acid (1.0 ml/ kg) after drug vehicle pretreatments elicited a mean \pm SEM of 13.1 ± 4.1 writhes. The absolute number of control writhes elicited by acid after vehicle pretreatment in each group is reported in the legend of Figure 5.1. All 6 mu opioid receptor agonists produced a dose-dependent decrease in acid-stimulated stretching, and ED50 values are shown in Table 5.1.

Effects of Mu Agonists in the Assay of Acid-Depressed ICSS

Effects of the lactic acid noxious stimulus on ICSS. Figure 5.2 shows effects of the same noxious stimulus (IP injection of 1.8% lactic acid) on ICSS. During each test session, a “baseline” frequency-rate curve was determined before experimental treatments to permit determination of the MCR for that session. Over the course of the entire study, the mean \pm SEM MCR was 61.49 ± 8.91 stimulations per trial. Reinforcement rates during each frequency trial of a session were then expressed as a percentage of that session’s MCR, and the average frequency-rate curve for all studies with drug vehicle + acid vehicle is shown in Figure 2. Maximum reinforcement rates

were usually observed at the highest stimulation frequencies (112-158 Hz), and responding generally decreased in a frequency-dependent manner. Administration of 1.8% lactic acid depressed ICSS, producing a rightward shift in the frequency-rate curve. Figure 5.2 also shows summary data for the total number of stimulations delivered across all 10 frequencies during each component. The overall mean \pm SEM baseline number of stimulations per component for all rats in the study was 319 ± 79.5 . Total ICSS after treatment with vehicle + acid vehicle was nearly identical to baseline pre-drug ICSS, but acid treatment decreased the number of stimulations per component. This acid-induced depression of ICSS provided a measure of pain-related behavioral depression, and opioids were evaluated for their ability to block this acid-induced depression of ICSS.

Methadone and fentanyl. Figure 5.3 shows that methadone and fentanyl dose-dependently and completely blocked 1.8% acid-induced depression of ICSS at or near doses that also facilitated control ICSS in the absence of the noxious acid stimulus. When administered as a pretreatment to acid vehicle, methadone doses of 0.032-0.32 mg/kg produced leftward shifts in the ICSS frequency-rate curve and significant facilitation of ICSS at intermediate frequencies of brain stimulation, whereas the highest dose of 1.0 mg/kg methadone only depressed ICSS at the highest two frequency (141-158 Hz) (Fig 5.3A). Similarly, when administered as a pretreatment to 1.8% lactic acid, methadone increased ICSS responding and ameliorated acid-induced depression of ICSS (Fig 5.3B). Significant increases in ICSS responding were observed after pretreatment with all methadone doses at a broad range of frequencies ranging from 71-112 Hz, and 0.1 mg/kg was the dose that produced the maximal attenuation of acid-

induced depression of ICSS. The highest dose of 1.0 mg/kg methadone also decreased ICSS at 158 Hz after acid pretreatment.

Pretreatment with fentanyl also non-selectively increased ICSS responding in the absence (Fig 5.3D) or presence of the acid noxious stimulus (Fig 5.3E). Doses of 0.01 and 0.032 mg/kg fentanyl significantly increased rates of reinforcement under both conditions across a broad range of frequencies from 79-112 Hz, and these effects of fentanyl are summarized in Figure 5.3F. Overall, methadone and fentanyl produced nonselective facilitation of ICSS in the absence or presence of acid, although both drugs were more efficacious to facilitate ICSS during acid treatment compared to acid vehicle treatment.

Morphine and hydrocodone. Figure 5.4 shows effects of morphine and hydrocodone on ICSS in the absence or presence of the acid noxious stimulus. When administered as a pretreatment to acid vehicle, morphine doses of 0.1-1.0 mg/kg had no significant effect on ICSS, but a higher dose of 3.2 mg/kg significantly depressed high rates of ICSS maintained by some high brain stimulation frequencies (Fig. 5.4A). When administered as a pretreatment to lactic acid, morphine doses of 0.1-1.0 mg/kg dose-dependently blocked acid-induced depression of ICSS; the higher dose of 3.2 mg/kg also attenuated acid-induced depression of ICSS, though to a lesser degree than 1.0 mg/kg (Fig. 5.4B). In contrast to morphine, hydrocodone doses of 0.1-3.2 mg/kg dose dependently facilitated ICSS in the absence of the noxious stimulus (Fig. 5.4D) and also dose-dependently blocked acid-induced depression of ICSS (Fig. 5.4E). No dose of hydrocodone depressed ICSS at any frequency in the absence or presence of the noxious stimulus. Overall, both morphine and hydrocodone blocked acid-induced

depression of ICSS, although with hydrocodone, this was accompanied by facilitation of ICSS in the absence of the noxious stimulus (Fig. 5.4C and 5.4F).

Buprenorphine and nalbuphine. Figure 5.5 shows that, similar to hydrocodone, the lower efficacy mu agonists buprenorphine and nalbuphine produced dose-dependent facilitation of ICSS in the absence of the noxious stimulus (Fig. 5.5A and 5.5D) and also dose-dependently blocked acid-induced depression of ICSS (Fig. 5.5B and 5.5E). No dose of buprenorphine or nalbuphine depressed ICSS at any frequency in the absence or presence of the noxious stimulus. Summary data for buprenorphine and nalbuphine are shown in Figures 5.5C and 5.5F, respectively.

Opioid ED50 values to block acid-induced depression of ICSS. Figure 6 shows effects of all 6 opioids expressed as “percent blockade” in the assay of acid-induced depression of ICSS. For the highest efficacy drugs methadone, fentanyl and morphine, an inverted U-shape curve was produced, such that peak blockade of acid-induced ICSS depression was achieved with intermediate doses (Fig. 5.6A). Conversely, hydrocodone, buprenorphine and nalbuphine produced dose-dependent blockade of acid-induced depression of ICSS across all doses examined (Fig. 5.6B). Table 5.1 shows ED50 values for the effects of each drug (derived from the ascending limbs of the dose-effect curves for methadone, fentanyl and morphine).

Effects of Noxious Stimulus Intensity

Figure 5.7 shows the effect of different concentration of lactic acid on acid-stimulated writhing and acid-induced depression of ICSS. Vehicle produced 2.57 ± 1.72 stretches during the 30-min observation period, and vehicle did not facilitate or depress

ICSS compared to baseline. Lactic acid produced a bitonic effect on acid-stimulated stretching, such that 0.56% and 5.6% acid did not produce significant stimulation of stretching relative to vehicle, whereas 1.8% acid did produce significant stimulation of stretching (Fig. 5.7A). Because the high concentration of 5.6% lactic acid did not stimulate a significant stretching response, opioid effects were not examined. On the other hand, lactic acid produced a concentration-dependent depression of ICSS that was significant after 1.8 and 5.6% acid (Fig. 5.7B). Table 5.1 shows that increasing the noxious stimulus intensity to 5.6% lactic acid produced a decrease in the potency of both methadone (approximately 10-fold) and nalbuphine (approximately 15-fold) to block acid-induced depression of ICSS.

5.4. Summary

This study examined the effects of mu opioid agonists in assays of acid-stimulated stretching and acid-depressed ICSS in rats. Two independent variables were manipulated in each assay: the efficacy of mu agonists at the mu receptor, and the noxious stimulus intensity. Intraperitoneal injection of lactic acid produced concentration-dependent decrease in ICSS, and an inverted U-shape stimulation of stretching with a peak effect after 1.8% lactic acid. Pretreatment with mu agonists blocked acid-stimulated stretching and acid-induced depression of ICSS in the presence of 1.8% acid, regardless of the efficacy of the agonist. Increasing the intensity of the noxious stimulus decreased the potency of high and low mu opioid agonists to block acid-induced depression of ICSS. In the absence of the noxious stimulus and consistent with previous findings (Altarifi *et al.*, 2012), mu agonists tended to produce facilitation of

ICSS, which did not vary systematically as a function of drug efficacy at the mu receptor. Overall, clinically used mu opioid analgesics are effective to produce antinociception in assays of pain-stimulated and pain-depressed behaviors.

Figure 5.1

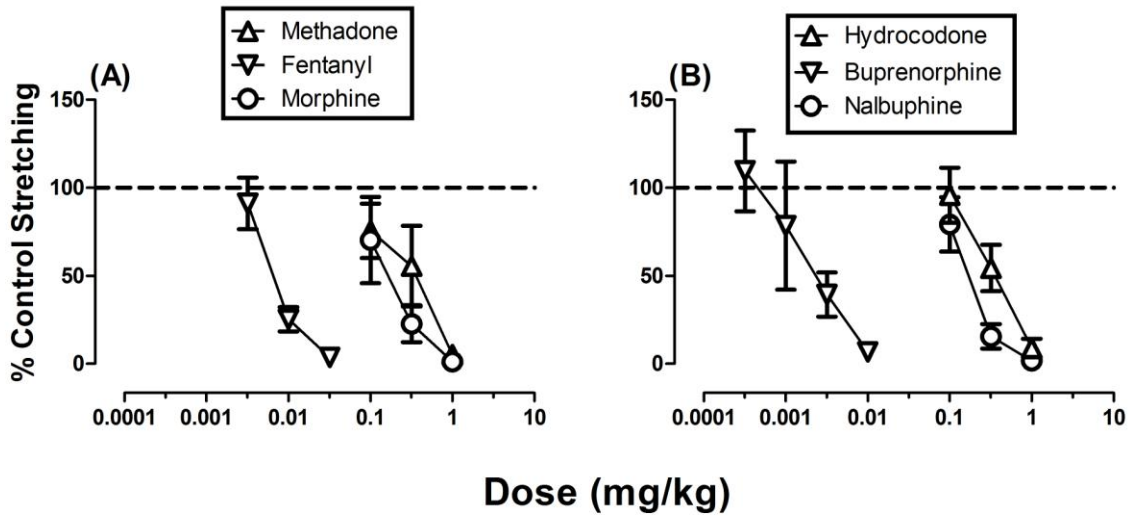


Figure 5.1. Effects of mu opioid agonists in the assay of acid-stimulated stretching. Abscissae: Dose in mg/kg. Ordinates: Percent control stretches. All points show mean data \pm SEM from 5 to 8 rats, and ED50 values are reported in Table 5.1. The mean \pm SEM number of control stretches for each group were as follows: methadone, 13.3 ± 4.3 ; fentanyl, 13.9 ± 2.7 ; morphine, 14.0 ± 7.6 ; hydrocodone, 12.7 ± 3.1 ; buprenorphine, 12.3 ± 4.1 ; nalbuphine, 12.9 ± 2.8 .

Figure 5.2

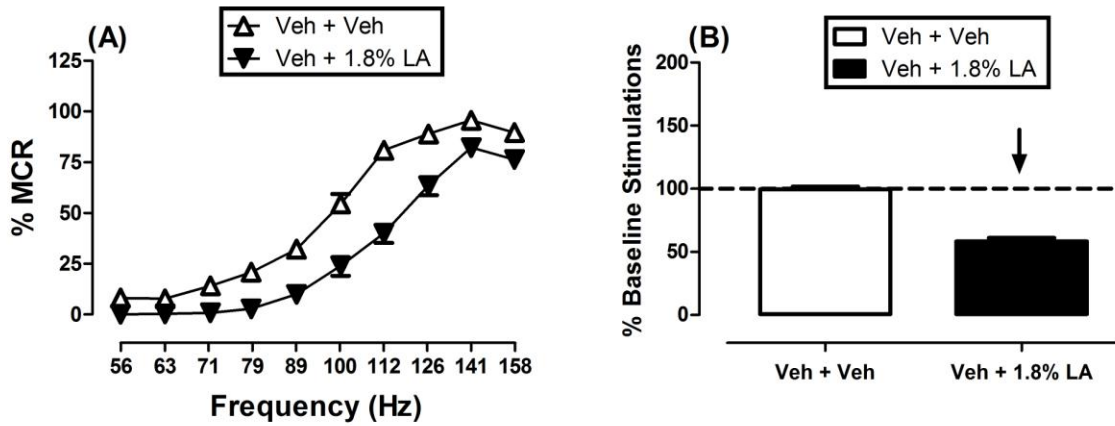


Figure 5.2. Depression of ICSS by 1.8% lactic acid. Left panel (A) compares effects of pretreatment with vehicle + vehicle (Veh + Veh) and vehicle + 1.8% lactic acid (Veh+ 1.8% LA) on full frequency-rate curves for all 35 rats used in the first phase of ICSS experiments. Abscissa: Frequency of electrical brain stimulation in Hz. Ordinate: ICSS rate expressed as percent maximum control response rate (%MCR). Two-way ANOVA indicated a significant main effect of frequency [$F(9,306) = 295.4, P < .001$] and acid treatment [$F(1,34) = 86.8, P < .001$], and the interaction was also significant [$F(9,306) = 10.2, P < 0.001$]. The acid noxious stimulus significantly depressed ICSS at all frequencies (Holm-Sidak post hoc test, $P < .05$). Right panel (B) shows summary data for lactic acid effects on the total number of stimulations per component. Abscissa: Pretreatment conditions. Ordinate: Percent baseline number of stimulations per component. The downward arrow indicates that lactic acid produced a significant decrease in ICSS at 1 or more frequencies in the full frequency-rate curve.

Figure 5.3

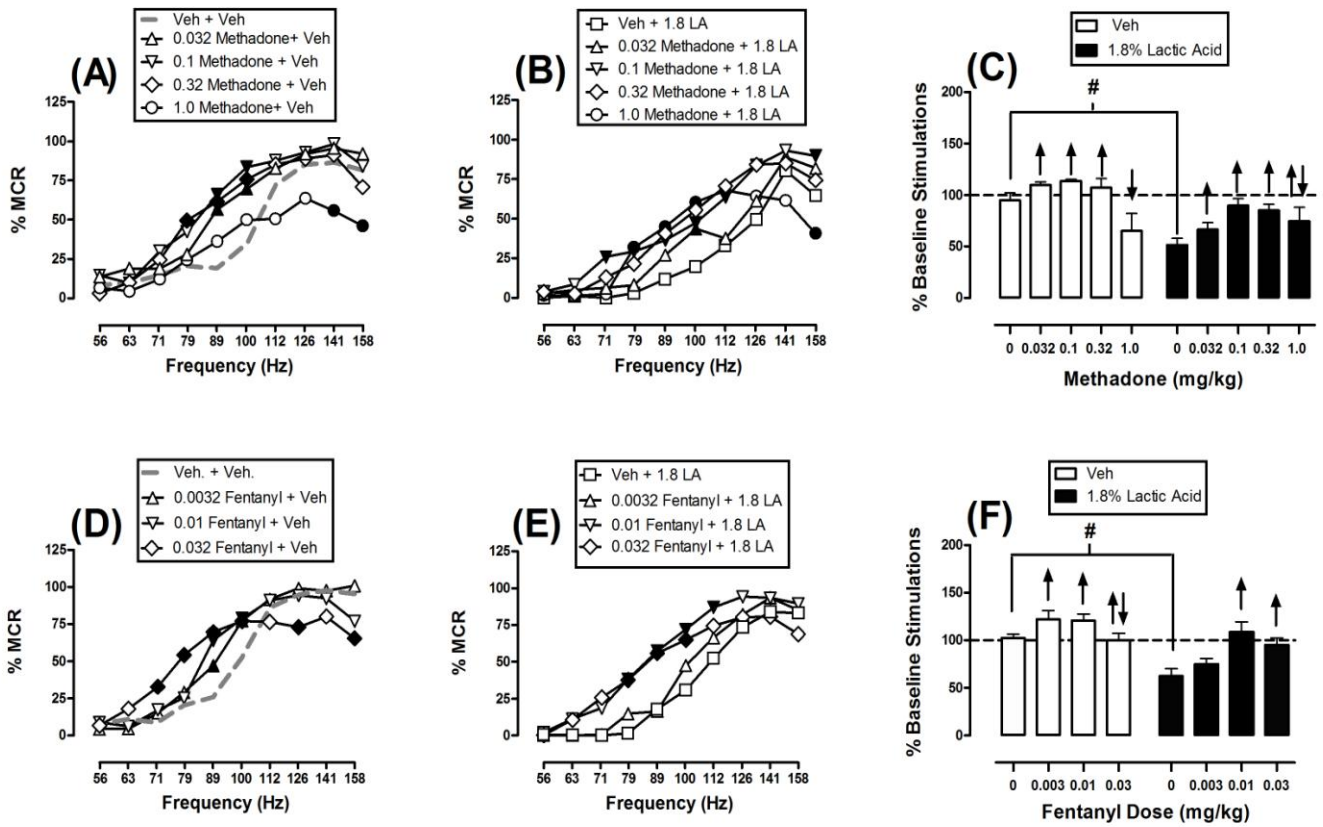


Figure 5.3. Effects of the methadone (panels A–C, N = 6) and fentanyl (panels D–F, N = 5) on control and 1.8% acid-depressed ICSS. Left and center panels show drug effects on full frequency-rate curves when drugs were administered as a pretreatment to vehicle (Left panels A, D) or 1.8% lactic acid (center panels B, E). Abscissae: Frequency of electrical brain stimulation in Hz. Ordinates: Percent maximum control response rate (%MCR). Filled symbols indicate a significant difference from Veh+Veh (A, D) or Veh+LA (B, E) (Holm-Sidak post hoc test, $P < .05$). Right panels (C, F) show summary data for drug effects on the total number of stimulations per component when drugs were administered as a pretreatment to vehicle (open bars) or acid (filled bars), and # signs indicate significant acid-induced depression of ICSS under drug vehicle conditions (paired t-test). Abscissae: Dose of drug in mg/kg. Ordinate: Percent baseline number of stimulations per component. Upward/downward arrows indicate that the drug dose produced a significant increase/decrease in ICSS at 1 or more frequencies in the full frequency-rate curve. Statistical results for 2-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,45) = 41.2, P < .001$], dose [$F(4,20) = 5.9, P = .003$], and interaction [$F(36,180) = 3.0, P < 0.001$]. (B) Significant main effect of frequency [$F(9,45) = 41.2, P < .001$], dose [$F(4,20) = 3.8, P = .018$], and interaction [$F(36,180) = 4.2, P < 0.001$]. (D) Significant main effect of frequency [$F(9,36) = 72.8, P < .001$], but not dose [$F(3,12) = 1.1, P = 0.384$]; the interaction was significant [$F(27,108) = 4.3, P < 0.001$]. (E) Significant main effect of frequency [$F(9,36) = 63.5, P < .001$] and dose [$F(3,12) = 6.1, P = 0.009$] and [$F(27,108) = 1.7, P = .030$].

Figure 5.4

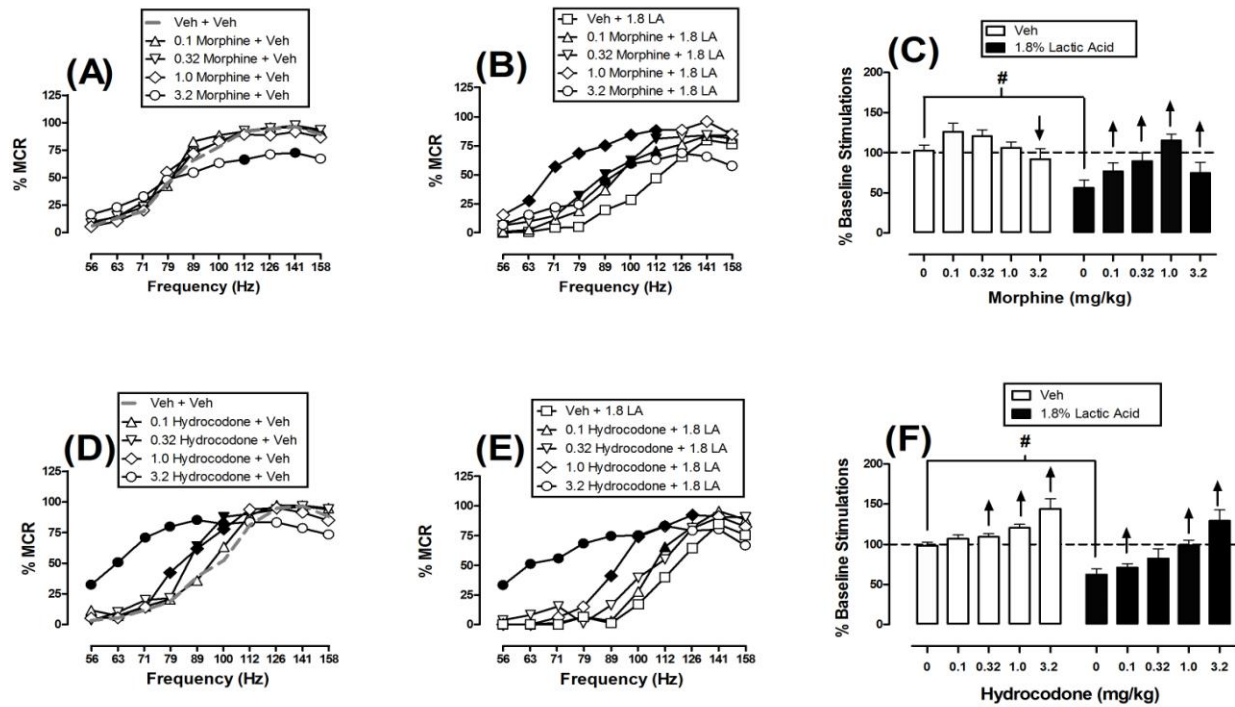


Figure 5.4. Effects of morphine (panels A–C, N = 6) and hydrocodone (panels D–F, N = 6) on control and 1.8% acid-depressed ICSS. Details as in Figure 5.3. Statistical results for 2-way ANOVA of full frequency-rate curves are as follows: (A) Significant main effect of frequency [$F(9,45) = 91.9, P < .001$], but not dose [$F(4,20) = 1.2, P = 0.356$]; the interaction was significant [$F(36,180) = 2.2, P < 0.001$]. (B) Significant main effects of frequency [$F(9,45) = 56.8, P < .001$], dose [$F(4,20) = 8.2, P < 0.001$], and interaction [$F(36,180) = 2.7, P < 0.001$]. (D) Significant main effect of frequency [$F(9,45) = 71.0, P < .001$], dose [$F(4,20) = 8.0, P < .001$], and interaction [$F(36,180) = 5.6, P < .001$]. (E) Significant main effect of frequency [$F(9,45) = 70.0, P < .001$], dose [$F(4,20) = 9.4, P < 0.001$], and interaction [$F(36,180) = 5.8, P < .001$].

Figure 5.5

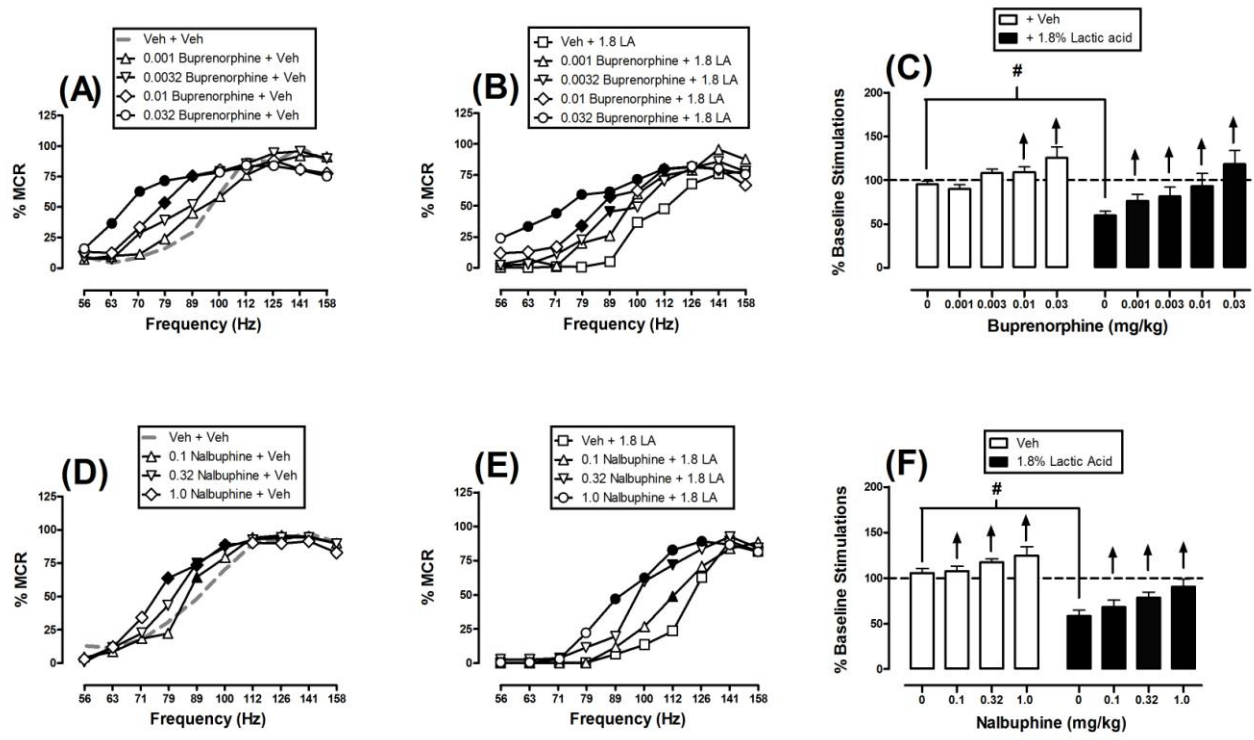


Figure 5.5. Effects of buprenorphine (panels A–C, N = 6) and nalbuphine (panels D–F, N = 6) on control and 1.8% acid-depressed ICSS. Details as in Figure 5.3.

Statistical results for 2-way ANOVA of full frequency-rate curves are as follows: (A)

Significant main effect of frequency [$F(9,45) = 70.6, P < .001$], dose [$F(4,20) = 2.9, P =$

.049], and interaction [$F(36,180) = 3.8, P < .001$]. (B) Significant main effects of

frequency [$F(9,45) = 37.9, P < .001$], dose [$F(4,20) = 4.2, P = 0.012$], and interaction

[$F(36,180) = 2.1, P < 0.001$]. (D) Significant main effect of frequency [$F(9,45) = 91.5, P$

$< .001$], but not dose [$F(3,15) = 1.5, P = 0.261$]; the interaction was significant

[$F(27,135) = 2.9, P < .001$]. (E) Significant main effect of frequency [$F(9,45) = 152.2, P <$

.001], dose [$F(3,15) = 5.1, P = 0.012$], and interaction [$F(27,135) = 2.9, P < .001$].

Figure 5.6

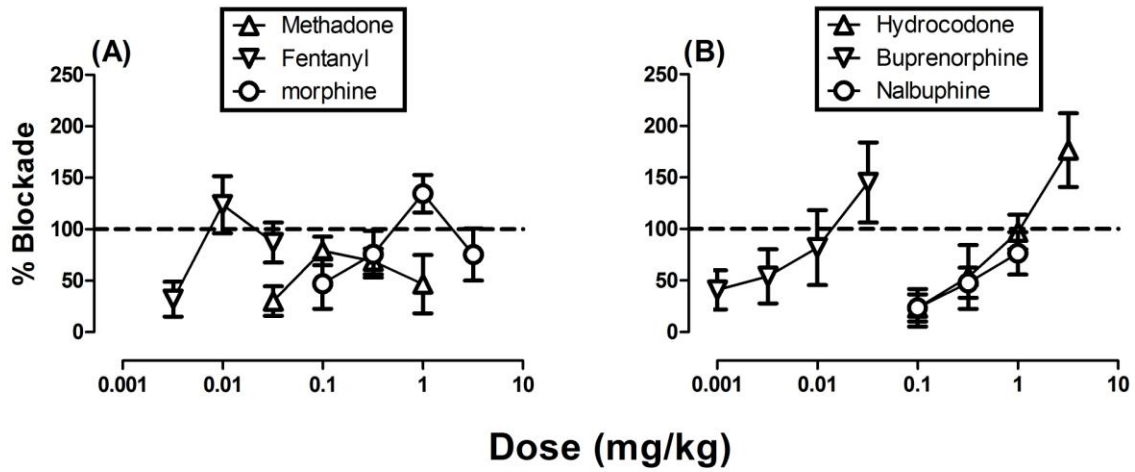


Figure 5.6. Dose-effect curves for mu agonist blockade of acid-induced depression of ICSS. Abscissae: Dose in mg/kg. Ordinates: Percent blockade of acid-induced depression of ICSS, calculated as described in Methods. All points show mean data \pm SEM from 5 to 6 rats, and ED50 values are reported in Table 5.1.

Figure 5.7

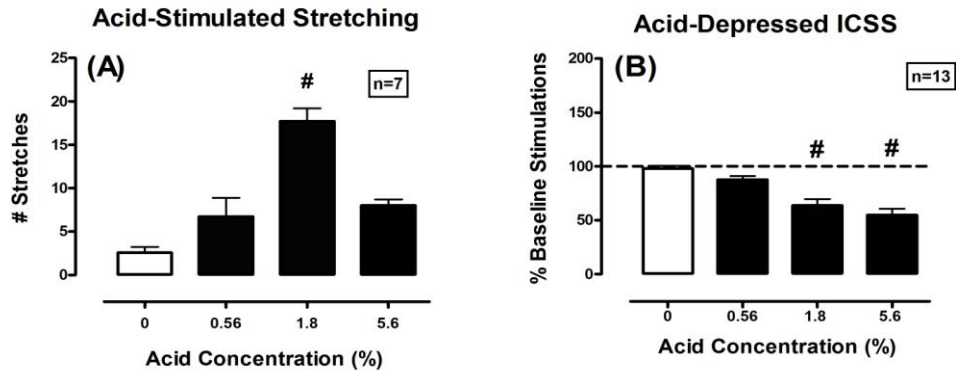


Figure 5.7. Effect of acid concentration on acid-stimulated stretching (left panel) and acid-depressed ICSS (right panel). Abscissae: lactic acid concentration administered IP; Left ordinate: number of stretches during the 30-min observation period; Right ordinate: Percent baseline number of stimulations per component. One-way ANOVA (with Dunnett post-hoc test; $P < 0.05$) revealed the following results: (A) significant effect of concentration [$F(6,18) = 16.7, P < 0.001$]. (B) significant effect of acid concentration [$F(12,36) = 22.3, P < 0.001$]. # indicate conditions under which total number of stimulations was significantly different from 0% (vehicle).

Table 5.1. ED50 Values in mg/kg (95% Confidence Limits) for mu opioid agonists to produce antinociception in the assays of acid-stimulated stretching or acid-induced depression of ICSS.

	Acid-Stimulated Stretching	Acid-Depressed ICSS
1.8% Lactic Acid		
Methadone	0.272 (0.145-0.511)	0.051 (0.030-0.085)*
Fentanyl	0.008 (0.006-0.011)	0.004 (0.003-0.006)*
Morphine	0.171 (0.097-0.301)	0.124 (0.070-0.218)
Hydrocodone	0.343 (0.236-0.501)	0.239 (0.161-0.355)
Buprenorphine	0.002 (0.001-0.005)	0.004 (0.002-0.008)
Nalbuphine	0.217 (0.152-0.309)	0.328 (0.130-0.826)
5.6% Lactic Acid		
Methadone	Not Tested	0.51 (0.08-3.46)
Nalbuphine	Not Tested	4.90 (1.52-15.86)

* Indicates significantly different from acid-stimulated stretching as indicated by non-overlapping confidence limits

CHAPTER SIX

Morphine antinociception is resistant to tolerance in an assay of pain-depressed intracranial self-stimulation

6.1. Introduction

Data from chapter 4 show that repeated morphine produced tolerance to its own rate-decreasing effects. If rate-decreasing effects contribute to morphine-induced antinociception in assays of pain-stimulated behavior, then repeated morphine might also produce tolerance to morphine effects in these assays. Conversely, if rate-decreasing effects do not contribute to morphine antinociception in assays of pain-depressed behavior, then repeated morphine might not produce tolerance to morphine antinociception in these assays; rather, repeated morphine might even enhance antinociception in these assays by producing tolerance to rate-decreasing effects that initially oppose and limit expression of antinociception. Thus, the hypothesis was that morphine would maintain or increase its antinociceptive potency in the assay of acid-induced depression of ICSS, but that tolerance would develop to morphine antinociception in the assay of acid-stimulated stretching.

6.2. Methods

6.2.1. Subjects

Subjects are similar to those described on section 2.2.1.

6.2.2. Assay of intracranial self-stimulation

Intracranial self-stimulation electrode implantation and behavioral procedure are similar to those described on section 2.2.2.

Testing: Once training and habituation to saline injections were completed, all rats received a single injection of 1.8% lactic acid to confirm sensitivity to acid-induced depression of ICSS prior to further testing. Next, “pre-drug baseline” sessions were conducted over a period of 3 consecutive days to establish baseline ICSS performance before administration of any dose of morphine. Each pre-drug baseline session consisted of 3 components as described in section 2.2.2, after which chronic treatment was initiated. Rats were divided into two groups that received either repeated morphine or repeated vehicle for 7 consecutive days. Rats receiving repeated morphine were treated with 3.2 mg/kg/day on days 1 and 2, 5.6 mg/kg/day on days 3 and 4, and 10 mg/kg/day on days 5, 6, and 7. The control group received daily vehicle (saline) injections. Three ICSS components were conducted before each daily injection, and two additional ICSS components were conducted beginning 30 min after each injection. On days 8, 10, 12, and 14, all animals in both groups were tested with a sequence of four treatments: (1) morphine vehicle + acid vehicle, (2) morphine vehicle + 1.8% lactic acid, (3) 1.0 mg/kg morphine + acid vehicle”, or (4) 1.0 mg/kg morphine + 1.8% lactic

acid. Morphine or its vehicle was administered 30 min before acid or its vehicle, and treatment order was randomized in a Latin-square design across animals. On each test day, ICSS was evaluated during 3 baseline components, followed immediately by subcutaneous treatment with 1.0 mg/kg morphine or its vehicle, after which subjects were returned to their home cages. After 30 min, subjects were treated intraperitoneally with 1.8% lactic acid or its vehicle and returned to the ICSS chambers for 2 ICSS test components. Immediately after testing, subjects in the chronic morphine group received a supplemental injection of morphine (either 9 or 10 mg/kg) to maintain the total daily dose of 10 mg/kg/day. In addition, on the non-test days (i.e. Days 9, 11, and 13), animals were maintained on 10 mg/kg/day morphine or vehicle, and ICSS components were conducted before and after injections as on Days 1-7. Table 6.1 summarizes all the treatments over the two-week chronic experiment.

Data analysis. The primary dependent measure 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 3 pre-drug baseline days before chronic treatment was initiated (6 total components). Baseline ICSS values in the chronic saline and morphine groups were compared by t-test. Data collected during chronic treatment and testing were then normalized to these baselines using the equation $\% \text{ Baseline Stimulations per Component} = (\text{Stimulations per Test Component} / \text{Baseline}) \times 100$. Statistical analysis focused on data from the test components on Days 8, 10, 12 and 14. Data from these 2 test components were

averaged within each rat to yield average % Baseline Stimulations per Component for each treatment in each rat. Data for each treatment were then averaged across rats and compared by two-way ANOVA, with acute treatment as one factor (1.0 mg/kg morphine or vehicle + 1.8% acid or vehicle), and chronic treatment as the other factor (10 mg/kg/day morphine or vehicle). A significant ANOVA was followed by the Bonferroni post-hoc test, and the criterion for significance was set a priori at $P < 0.05$.

6.2.3 Assay of acid-stimulated stretching

To evaluate stretching behavior, rats were placed into acrylic test chambers (31.0 X 20.1 X 20.0 cm) for 30-minute observation periods. A stretch was operationally defined as a contraction of the abdomen followed by extension of the hind limbs, and the number of stretches during the observation period was counted. Initially, all rats were evaluated for 30 min after a single injection of 1.8% lactic acid to confirm sensitivity to acid-stimulated stretching prior to further testing. Subsequently, rats were divided into two groups that received either repeated morphine or repeated vehicle for 7 consecutive days. As in the assay of acid-depressed ICSS, rats receiving repeated morphine were treated with 3.2 mg/kg/day on days 1 and 2, 5.6 mg/kg/day on days 3 and 4, and 10 mg/kg/day on days 5, 6, and 7, whereas the control group received daily vehicle (saline) injections. To mimic handling conditions in ICSS rats, subjects were placed into a clean acrylic chamber for 30 minutes before each daily injection, returned to their home cage for 30 min after each daily injection, and then placed back into the acrylic chamber for another 30 minutes. On days 8, 10, 12, and 14, all rats in both groups were tested with the same sequence of treatments that was tested in ICSS rats:

(1) morphine vehicle + acid vehicle, (2) morphine vehicle + 1.8% lactic acid, (3) 1.0 mg/kg morphine + acid vehicle, or (4) 1.0 mg/kg morphine + 1.8% lactic acid. On each test day, rats were placed into the acrylic observation chamber for 30 min, followed immediately by subcutaneous treatment with 1.0 mg/kg morphine or its vehicle, and subjects were then returned to their home cages. After 30 min, subjects were treated intraperitoneally with 1.8% lactic acid or its vehicle and returned to the chamber for observation of stretching. Immediately after testing, subjects in the chronic morphine group received a supplemental injection of morphine to maintain the total daily dose of 10 mg/kg/day. In addition, on the non-treatment days (i.e. Days 9, 11, and 13), rats were maintained on 10 mg/kg/day morphine or vehicle and exposed to the acrylic chamber before and after injections as on Days 1-7.

Data Analysis. The primary dependent variable was the number of stretches observed during the 30-minute observation period after treatments on test days 8, 10, 12 and 14. Data for each treatment were averaged across rats and compared by two-way ANOVA, with acute treatment as one factor (1.0 mg/kg morphine or vehicle + 1.8% acid or vehicle), and chronic treatment as the other factor (10 mg/kg/day morphine or vehicle). A significant ANOVA was followed by the Bonferroni post-hoc test, and the criterion for significance was set a priori at $P < 0.05$.

6.2.4. Drugs

Morphine sulfate was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland, USA) and prepared in sterile saline for subcutaneous injection. Lactic acid was purchased from Sigma Aldrich (St. Louis, MO)

and diluted in sterile water for intraperitoneal injection. All injections were delivered in a volume of 1.0 ml/kg.

6.3. Results

Assay of acid-stimulated stretching. Figure 6.1 shows the effects of different acute treatments on stretching behavior in rats treated chronically with vehicle or morphine. In the chronic vehicle group, 1.8% lactic acid stimulated a stretching response, and 1.0 mg/kg morphine blocked acid-stimulated stretching while having no effect on stretching in the absence of the noxious stimulus. In the chronic morphine group, 1.8% lactic acid stimulated a significantly greater number of stretches than in the chronic vehicle group. Moreover, tolerance developed to the antinociceptive effects of 1.0 mg/kg morphine, such that morphine no longer produced a significant decrease in stretching. In addition, stretching after treatment with 1.0 mg/kg morphine + acid was significantly greater in the chronic morphine group than in the chronic vehicle group.

Assay of acid-depressed ICSS. During baseline sessions, the mean \pm SEM total numbers of stimulations for the chronic vehicle group and chronic morphine group were 339.8 ± 37.8 and 312.9 ± 57.9 stimulations per component, respectively ($t=0.34$, not significantly different). Figure 6.2 shows the effects of different acute treatments on ICSS in rats treated chronically with vehicle or morphine. In the chronic vehicle group, 1.8% lactic acid significantly depressed ICSS, and 1.0 mg/kg morphine blocked acid-induced depression of ICSS while having no effect on ICSS in the absence of the noxious stimulus. In the chronic morphine group, 1.8% lactic acid also depressed ICSS.

Although mean rates of ICSS were lower after acid in the chronic morphine group than in the chronic vehicle group, this difference was not statistically significant. Tolerance did not develop to morphine antinociception in the chronic morphine group. Thus, as in the chronic vehicle group, 1.0 mg/kg morphine blocked acid-induced depression of ICSS at a dose that did not alter ICSS in the absence of the noxious stimulus. ICSS after 1.0 mg/kg morphine + acid was not different in the chronic vehicle and chronic morphine groups.

6.4. Summary

This study examined the hypothesis that morphine would maintain or increase its antinociceptive potency in the assay of acid-induced depression of ICSS, but that tolerance would develop to morphine antinociception in the assay of acid-stimulated stretching. My results were consistent with this hypothesis and two main findings summarize my results. First, repeated morphine administration exacerbated acid-induced stretching and acid-induced depression of ICSS when lactic was administered during morphine abstinence. Second, morphine at a dose of 1.0 mg/kg was effective to block acid-induced stretching and acid-depressed ICSS in the repeated-vehicle treated group. However, repeated morphine administration produced tolerance to the antinociceptive effect of 1.0 mg/kg morphine in the assay of acid-stimulated stretching, but not in the assay of acid-depressed ICSS.

Figure 6.1

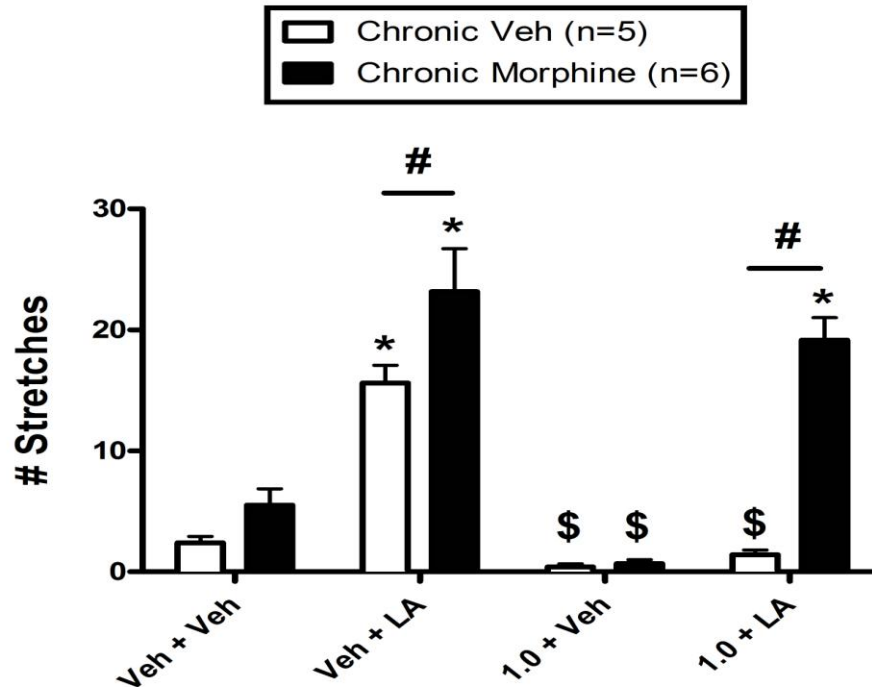


Figure 6.1. Effects of different acute treatments on stretching behavior in rats treated chronically with vehicle or morphine. Subjects were treated repeatedly with saline (open bars) or morphine (filled bars). Horizontal axis: acute treatment of 1.0 mg/kg morphine or vehicle + 1.8% lactic acid or vehicle. Vertical axis: number of stretches during 30-min observation period. Two-way ANOVA showed that there was significant main effect of acute treatment [$F=45.4$; $P<0.001$], significant main effect of chronic group [$F=34.1$; $P<0.001$], and significant treatment x chronic group interaction [$F=9.7$; $P<0.001$]. * Asterisks indicate treatments that were significantly different from vehicle + vehicle within the same group. \$ Dollar signs indicate treatments that were significantly different from vehicle + 1.8% lactic acid within the same group. # indicates significance between groups after the same treatment.

Figure 6.2

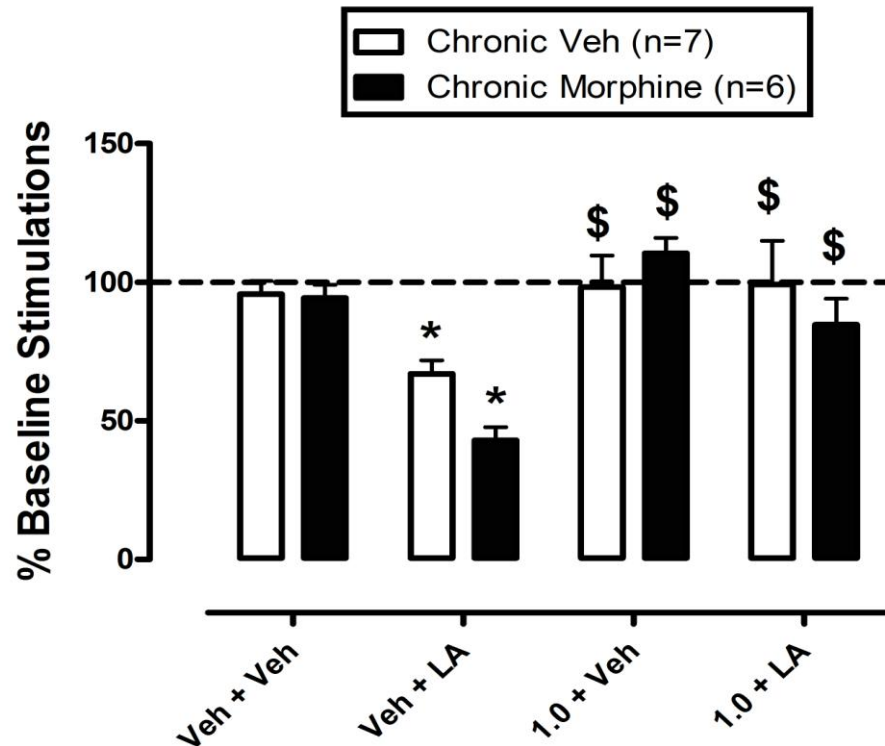


Figure 6.2. Effects of different acute treatments on ICSS behavior in rats treated chronically with vehicle or morphine. Subjects were treated repeatedly with saline (open bars) or morphine (filled bars). Horizontal axis: acute treatment of 1.0 mg/kg morphine or vehicle + 1.8% lactic acid or vehicle. Vertical axis: percent baseline number of stimulations per component. Two-way ANOVA showed that there was a significant main effect of acute treatment [$F=11.8$; $P<0.001$], no significant main effect of chronic group [$F=1.2$; $P=0.281$], and no significant treatment x chronic group interaction [$F=1.6$; $P=0.214$]. * Asterisks indicate treatments that were significantly different from vehicle + vehicle within the same group. \$ Dollar signs indicate treatments that were significantly different from vehicle + 1.8% lactic acid within the same group.

Table 6.1. Summary table representing the daily treatments for each group in the study. Morphine doses are shown in mg/kg. “Test” indicates a treatment with (1) morphine vehicle + acid vehicle, (2) morphine vehicle + 1.8% lactic acid, (3) 1.0 mg/kg morphine + acid vehicle, or (4) 1.0 mg/kg morphine + 1.8% lactic acid. Subjects in the chronic morphine group also received a supplemental treatment of morphine at the end of the test session on that day to maintain the total dose at 10 mg/kg/day. The chronic vehicle group did not receive any supplemental injections on test days.

Day	Chronic vehicle group	Chronic Morphine Group	
	Daily treatment	Daily treatment	Supplemental treatment
1-2	Saline	3.2 morphine	N/A
3-4	Saline	5.6 morphine	N/A
5-7	Saline	10 morphine	N/A
8	Test	Test	9-10 morphine
9	Saline	10 morphine	N/A
10	Test	Test	9-10 morphine
11	Saline	10 morphine	N/A
12	Test	Test	9-10 morphine
13	Saline	10 morphine	N/A
14	Test	Test	9-10 morphine

CHAPTER SEVEN

Discussion

7.1. General summary

This project tested a series of independent variables to evaluate effects of mu opioid receptor agonists on ICSS. Some of these independent variables include drug efficacy, dose, pretreatment time, and repeated administration of morphine. Opioids' effects on ICSS were evaluated in two phases:

- 1) Phase 1: Experiments were conducted in the absence of any noxious stimulus.
- 2) Phase 2: opioid effects were evaluated in the presence of an acute visceral noxious stimulus (intraperitoneal injection of dilute lactic acid)

The discussion that follows will describe and interpret mu agonist effects on ICSS. As a prelude to this discussion, it should be noted that our "frequency-rate" ICSS procedure used a wide range of brain stimulation frequencies to maintain a wide range of baseline behavioral rates. Drug effects on these different baseline rates of behavior will be described using two terms. "Rate-decreasing effects" describes treatment-

induced decreases in high rates of ICSS maintained by high brain stimulation frequencies. “Rate-increasing effects” will be used to describe treatment-induced increases in low rates of ICSS maintained by low brain stimulation frequencies. Importantly, both effects can occur simultaneously, and these two effects can be differentially modulated.

Phase 1. Four major findings summarize data from this phase. First, effects of acute opioid administration in naïve subjects are efficacy dependent, such that high-efficacy ligands produce dose-dependent rate-decreasing effects, while low-efficacy ligands produce mild and inconsistent rate-increasing effects and antagonize the rate-decreasing effects of high-efficacy agonists. Second, repeated administration of morphine produces tolerance to its rate-decreasing effect and cross-tolerance to the rate-decreasing effects of other high-efficacy ligands, such as methadone and fentanyl. Third, repeated morphine administration does not produce clear tolerance to its rate-increasing effects, but rather enhances the expression of abuse-related rate-increasing effects of high- and low-efficacy mu agonists. Finally, abstinence from morphine after repeated administration induces withdrawal-related decrease in ICSS. Collectively, these findings suggest that mu opioid agonist effects on ICSS are dependent on history of opioid administration, and abuse liability of these drugs is enhanced after repeated opioid administration.

Phase 2. Results from this phase can be summarized into three major findings. First, mu receptor agonists blocked pain-induced depression of ICSS; there was little effect of efficacy between drugs with the exception that high-efficacy drugs produced inverted U-

shaped dose-effect curves, and also tended to be more potent to block acid-depressed ICSS compared to acid-stimulated stretching. Second, both the high-efficacy agonist methadone and the low-efficacy agonist nalbuphine retained their antinociceptive effects in the presence of a high intensity noxious stimulus, although the potency of both drugs was reduced. Third, morphine antinociception was resistant to tolerance in the assay of acid-depressed ICSS in comparison to the assay of acid-stimulated stretching. Taken together, these findings suggest that mu agonists across a broad range of efficacies produce antinociception in this assay of acid-depressed ICSS, and that morphine antinociception in this assay is resistant to tolerance. The potential relationship will be discussed below between (a) increased expression of ICSS facilitation after repeated morphine, and (b) resistance to antinociceptive tolerance in the assay of acid-depressed ICSS.

7.2. Effects of acute morphine (phase 1)

Results in chapter 2 agree with previous studies in finding that acute morphine effects on ICSS can display a biphasic time course characterized by an initial decrease followed by a subsequent increase in rates of ICSS (Adams *et al.*, 1972; Lorens and Mitchell, 1973; Bermudez-Rattoni *et al.*, 1983). A similar biphasic time course was also produced by the mu agonist heroin (Koob *et al.*, 1975). In these earlier studies, baseline responding was maintained at relatively stable rates by constant intensities and frequencies of electrical stimulation. This study extends these findings by using a 'frequency–rate' procedure that generated a wide range of baseline response rates to systematically examine the rate dependency of acute morphine effects. This procedure

confirmed that early rate-decreasing effects of morphine were greatest for high baseline response rates maintained by high frequencies of stimulation, whereas later rate-increasing effects of morphine were most prominent for low to intermediate response rates maintained by low-to-intermediate frequencies of stimulation. Additional discussion of rate dependence is provided below.

In chapter 2, low morphine doses (1–3.2 mg/kg) produced small but significant increases in low ICSS rates during the first 30 min after morphine injection. This agrees with the finding that similarly low morphine doses at similarly short pretreatment times produced facilitation of ICSS in some (Kornetsky and Esposito, 1979; Carlezon and Wise, 1993; Jha *et al.*, 2004) but not all (Stratmann and Craft, 1997; Pereira Do Carmo *et al.*, 2009) studies that focused on low baseline ICSS rates maintained by threshold frequencies or intensities of stimulation. However, any facilitation of ICSS observed early in the time course of low-dose acute morphine is weaker and less consistent than the more robust facilitation of ICSS observed later in the time course of larger morphine doses (chapter 2; (Easterling and Holtzman, 1997; O'Neill and Todtenkopf, 2010)).

The biphasic time course of acute morphine effects on rates of ICSS is similar to the biphasic time course of morphine effects on rates of locomotor activity in rats (Vasko and Domino, 1978; Craft *et al.*, 2006). For example, morphine (1–10 mg/kg subcutaneously) produced initial dose-dependent decreases (approximately 30–90 min after injection) followed by later increases (approximately 2–5 h after injection) in horizontal activity in male rats relative to their saline-treated controls (Craft *et al.*, 2006). Moreover, the time course of morphine-induced depression of ICSS and locomotion corresponds to the time course of morphine-induced depression of some other

behaviors, such as thermal nocifensive behaviors in tail flick and hot-plate assays (Cicero *et al.*, 1996; Cicero *et al.*, 1997), and a stimulation of pain-related behaviors may emerge after antinociceptive effects have dissipated (i.e. opioid-induced hyperalgesia) (Chu *et al.*, 2008). Taken together, these findings provide evidence of relatively broad behavioral depressant effects of acute morphine that may be followed by later behavioral stimulant effects. Factors that underlie the emergence of morphine-induced stimulant effects are not known. One possibility is that acute tolerance develops to behavioral depressant effects, thereby unmasking behavioral stimulant effects. Section 7.4 discusses this possibility based on data collected from chronic studies.

7.3. Effects of drug efficacy (phase 1)

Drugs with varying efficacies at mu receptors can be tested to provide insight into the efficacy requirements of different effects produced by mu agonists. For example, low-efficacy mu agonists such as nalbuphine often produce antinociception against low- but not high-intensity noxious thermal stimuli, whereas higher efficacy mu agonists such as morphine and methadone are more likely to produce antinociception against both low- and high-intensity noxious stimuli (Morgan *et al.*, 1999; Negus and Mello, 1999; Cook *et al.*, 2000). Such data provide one source of evidence to suggest that antinociception against low-intensity noxious stimuli in conventional assays of pain-stimulated behavior has lower efficacy requirements than antinociception against high-intensity noxious stimuli.

In chapter 3, the low-efficacy mu agonist nalbuphine facilitated ICSS across a broad range of doses but produced little or no depression of ICSS, whereas the higher

efficacy mu agonist methadone produced biphasic facilitation and depression of ICSS. These findings are superficially consistent with lower efficacy requirements for facilitation than depression of ICSS. However, these results were collected in opioid-experienced rats, albeit using a twice-per-week testing regimen intended to minimize opioid tolerance. Given the known potential for opioid exposure to attenuate ICSS-depressing effects as shown in Chapter 2, a follow-up study was conducted with nalbuphine and methadone in opioid-naïve rats. Methadone was more potent in depressing ICSS and both drugs were less effective in facilitating ICSS in opioid-naïve than opioid-experienced rats. The differences in methadone effects between opioid-naïve and opioid-experienced rats suggests that even intermittent opioid exposure associated with twice-per-week testing was sufficient to produce tolerance to ICSS depression similar to that observed with more intensive daily regimens of morphine treatment (Chapter 2). Moreover, these results suggest that opioid exposure also augments expression of rate-increasing effects produced by low-efficacy mu agonists like nalbuphine that do not reliably facilitate ICSS in naïve rats. Overall, the limited and unreliably dose-dependent ability of nalbuphine to facilitate ICSS in rats with little or no history of opioid exposure fails to support the hypothesis that opioid-induced facilitation of ICSS has lower efficacy requirements at mu receptors than opioid-induced depression of ICSS. This conclusion was also supported by other experiments conducted in the same laboratory, which showed similar sensitivity of morphine-induced rate-increasing and rate-decreasing effects to antagonism by the irreversible mu antagonist β -funaltrexamine (Altarifi et al., 2012).

7.4. Effects of repeated morphine administration (phase 1)

7.4.1. Effects of morphine during repeated administration of morphine

In chapter 2, repeated morphine treatment reduced the initial rate-decreasing effects of morphine while producing earlier expression of rate-increasing effects. The onset of tolerance to the rate-decreasing effects of morphine seemed to be rapid. Informal analysis of data obtained during the acute-dosing phase of the study did not reveal a systematic effect of the dose order on rate-increasing versus rate-decreasing morphine effects (data not shown); however, ICSS was not significantly altered 30 min after 3.2 mg/kg morphine during the acute phase of the study but was significantly facilitated 30 min after administration of the same dose on day 1 of the chronic dosing phase of the study. This suggests that morphine exposure associated with intermittent dosing to determine the dose-effect curve during the acute-dosing phase of the study was sufficient to produce some degree of tolerance to the rate-decreasing effects of morphine, and the extent of this tolerance became more pronounced during the chronic-dosing phase. This agrees with the effects of repeated morphine from studies that used simpler ICSS procedures in which relatively constant baseline response rates were maintained by constant magnitudes of brain stimulation (Lorens and Mitchell, 1973). For example, Lorens and Mitchell 1973 administered morphine doses of 5, 10, or 20 mg/kg daily for 5 days in rats trained to respond for a single magnitude of brain stimulation. On the first day of treatment, all three doses produced initial rate-decreasing effects followed by later rate-increasing effects. However, as early as the third day of treatment,

doses of 5 and 10 mg/kg morphine ceased to produce rate-decreasing effects, and rate-increasing effects occurred with an earlier onset and similar duration of action. These results also agree with studies using more sophisticated procedures that showed reductions in initial rate-decreasing effects and/or increased expression of rate-increasing effects during repeated morphine treatment (Carlezon and Wise, 1993; Easterling and Holtzman, 1997; Craft *et al.*, 2001). This study adds to this literature by evaluating the effects of a broad range of morphine doses on responding maintained across a broad range of ICSS rates. Finally, the tolerance to morphine-induced rate-decreasing effects produced by repeated morphine in assays of ICSS is qualitatively similar to the rapid tolerance to morphine-induced rate-decreasing effects that develop in assays of locomotor activity (Babbini and Davis, 1972; Vasko and Domino, 1978; Smith *et al.*, 2009).

A parsimonious interpretation of these data is that morphine effects on ICSS reflect an integration of rate-decreasing and rate-increasing effects, and that tolerance to rate-decreasing effects results in an unmasking of rate-increasing effects. The mechanisms responsible for this tolerance are not known and may include processes of either pharmacodynamic or behavioral tolerance (Smith, 1979; Negus *et al.*, 2010). For example, it is well-established that tolerance can develop at different rates to different morphine effects, and this differential tolerance to behavioral effects is accompanied by differential tolerance to intracellular signaling in different mu opioid receptor populations in the brain. Thus, chronic morphine treatment selectively decreased mu agonist-stimulated G-protein activation in brainstem nuclei (including dorsal raphe nucleus and locus coeruleus) thought to contribute to rate-decreasing effects of opioids, but not in

the forebrain structures (including NA and amygdala) thought to contribute to the stimulant effects of opioids (Broekkamp *et al.*, 1976; Sim *et al.*, 1996). Overall, mu receptor populations mediating abuse-related facilitation of ICSS may be more resistant to tolerance during chronic opioid exposure than mu receptor populations mediating rate suppression. Increased expression of morphine-induced stimulant effects may involve not only a resistance to tolerance but also a sensitization of neural circuits that mediate these effects. For example, a regimen of repeated morphine administration similar to that used here increased expression of the GluR1 subunit of AMPA glutamate receptors in rat ventral tegmental area, an effect that could increase sensitivity of ventral tegmental area dopaminergic neurons to glutamatergic inputs (Fitzgerald *et al.*, 1996).

7.4.2. Cross-tolerance between opioids on ICSS

Chapter 4 examined the impact of graded morphine exposure on changes in ICSS produced by agonists with high efficacy (methadone), intermediate efficacy (fentanyl) or low efficacy (nalbuphine) at mu opioid receptors. There were three main findings. First, in agreement with previous results with morphine described in chapter 3, the higher efficacy mu agonists methadone and fentanyl produced primarily rate-decreasing effects in opioid-naïve subjects, whereas the low-efficacy mu agonist nalbuphine produced primarily rate-increasing effects that did not vary systematically as a function of dose. Second, repeated morphine produced cross tolerance to the rate-decreasing effects and enhanced expression of the rate-increasing effects of all three mu agonists. Lastly, the daily morphine dosing regimen used here produced withdrawal-associated decreases in baseline ICSS determined approximately 23 hrs after

morphine. Repeated morphine also enhanced rate-decreasing effects of the antagonist naltrexone. However, this evidence of opioid dependence and withdrawal was not sufficient to account for enhanced expression of mu agonist-induced rate-increasing effects. Taken together, these results provide further evidence to suggest that repeated opioid exposure increases the degree to which mu agonists produce abuse-related facilitation of ICSS.

Opioid effects in opioid-naïve rats. The constellation of rate-increasing and rate-decreasing effects produced by methadone, fentanyl and nalbuphine in opioid-naïve subjects in this study agrees with effects reported previously for these and other mu agonists that vary in efficacy at mu receptors (Chapter 3; Altarifi and Negus, 2011; Altarifi *et al.*, 2012). Specifically, rate-decreasing effects predominate for high-efficacy agonists; lower efficacy agonists produce weaker and more variable evidence of rate-increasing and rate-decreasing effects; and antagonists such as naltrexone fail to alter ICSS at doses that antagonize effects of mu agonists. The efficacy-dependent rate-decreasing effects of mu agonists in this ICSS procedure agree with the efficacy-dependent magnitude and/or variability in rate-decreasing effects of mu agonists in other assays of responding maintained by other reinforcers (e.g. food) under other schedules (Oliveto *et al.*, 1991; Pitts *et al.*, 1996). Moreover, in the ICSS literature, drug-induced facilitation of ICSS is often interpreted as evidence of an abuse-related effect, whereas drug-induced depression of ICSS is often interpreted as evidence of abuse-limiting dysphoric effects or motor impairment (Carlezon and Chartoff, 2007). From this perspective, the present results could be interpreted to suggest that abuse-limiting dysphoric and/or motor effects often predominate over abuse-related rewarding

effects of mu agonists in opioid-naïve rats. This finding in ICSS may be related to the observation that mu agonists are often more efficacious to produce dysphoric subjective effects and behavioral impairment than abuse-related euphoric effects in opioid-naïve/inexperienced human subjects (Lasagna *et al.*, 1955; Walker *et al.*, 2001).

Opioid effects in morphine-treated rats. Repeated morphine has been shown previously to produce tolerance to the ICSS-decreasing effects of morphine (Lorens and Mitchell, 1973; Altarifi and Negus, 2011), and the present study found that repeated morphine also produced cross-tolerance to the ICSS-decreasing effects of the other mu agonists methadone and fentanyl. This agrees with previous reports of morphine-induced cross tolerance to other effects of methadone and/or fentanyl, including their rate-decreasing effects in assays of schedule-controlled responding for food reinforcement (Picker *et al.*, 1991; Hughes *et al.*, 1995; Smith *et al.*, 1997) or their morphine-like discriminative stimulus effects (Young *et al.*, 1991; Walker *et al.*, 1997). Nalbuphine produced only small and inconsistent rate-decreasing effects before morphine treatment, and as a result, cross tolerance was difficult to assess. Nonetheless, the complete absence of nalbuphine-induced rate-decreasing effects during morphine treatment suggests that morphine also produced cross tolerance to any rate-decreasing effects of nalbuphine. Previous studies have failed to reveal cross tolerance between the rate-decreasing effects of morphine and nalbuphine in assays of schedule-controlled responding for food (Oliveto *et al.*, 1991; Picker and Yarbrough, 1991; Smith *et al.*, 1997), but nalbuphine rate-decreasing effects in these studies occurred only at high doses, were variable across subjects, and may have been associated with precipitated withdrawal during morphine treatment (see below). The

present findings agree with previous reports of cross tolerance between other effects of morphine and nalbuphine, such as discriminative stimulus effects (Walker *et al.*, 1997) and thermal antinociceptive effects (Gringauz *et al.*, 2001).

In addition to producing tolerance to mu agonist-induced rate-decreasing effects, repeated morphine treatment also enhanced expression of mu agonist-induced facilitation of ICSS. This agrees with previous studies reporting that repeated morphine enhances expression of its own ICSS-facilitating effects (Carlezon and Wise, 1993; Altarifi *et al.*, 2012), and extends this phenomenon to other mu agonists with a broad range of efficacies at mu receptors. As discussed above with morphine, unmasking of the rate-increasing effect after selective tolerance to rate-decreasing effects and/or sensitization to neural substrates that mediate rate-increasing effects are possible explanations for the enhanced expression of abuse-related rate-increasing effects of other mu agonists during morphine treatment. Regardless of the underlying mechanism, these data suggest a shift in morphine effects that favors expression of abuse-related rate-increasing effects relative to abuse-limiting rate-decreasing effects. This shift apparent in ICSS may be related to the finding that regimens of mu agonist exposure can also increase expression of abuse-related rewarding effects in preclinical assays of place conditioning (Lett, 1989; Shippenberg *et al.*, 1996), increase reinforcing effects in preclinical assays of self-administration (Thompson and Schuster, 1964; Yanagita, 1978; Carrera *et al.*, 1999; Negus and Rice, 2009), and increase expression of abuse-related subjective effects and reinforcing effects in humans (Comer *et al.*, 2010; Cooper *et al.*, 2012).

The regimen of repeated morphine treatment used in this study produced little evidence of tolerance to mu agonist-induced ICSS facilitation, even when the daily morphine dose was increased to 18 mg/kg/day. This dose was sufficient to produce signs of morphine dependence (see below), but even the low-efficacy mu agonist nalbuphine continued to produce significant ICSS facilitation across the entire dose range tested. Indeed, the only evidence for tolerance to rate-increasing effects was that a dose of 0.01 mg/kg fentanyl facilitated ICSS during treatment with 3.2 mg/kg/day morphine but not during treatment with 18 mg/kg/day morphine. It is possible that more intensive treatment regimens (e.g. higher morphine doses or longer treatment times) may have produced tolerance to rate-increasing effects. However, the present results suggest that mu agonist-induced facilitation of ICSS is relatively resistant to tolerance.

7.4.3. Role of morphine dependence and withdrawal

In addition to producing tolerance to morphine-induced rate-decreasing effects, repeated morphine also produced dependence as indicated by dose-dependent decreases in ICSS during spontaneous morphine withdrawal (figure 2.4; figure 4.1) and during precipitated withdrawal after administration of the mu opioid receptor antagonist naltrexone (figure 4.5). These findings agree with other studies reporting reductions in ICSS during spontaneous or antagonist-precipitated morphine withdrawal (Schaefer and Michael, 1983; Easterling and Holtzman, 1997; Liu and Schulteis, 2004). The absence of clear somatic withdrawal signs during spontaneous withdrawal further suggests that ICSS may be more sensitive than commonly assessed somatic signs to the impact of opioid withdrawal.

The withdrawal-associated depression of ICSS could be reversed by all three mu agonists (chapter 4). However, three findings suggest that enhanced expression of mu agonist-induced ICSS facilitation could not be attributed completely to reversal of morphine withdrawal. First, this enhanced expression of ICSS facilitation was observed during treatment with a lower dose of 3.2 mg/kg/day morphine, which did not produce evidence of dependence or withdrawal. Second, during treatment with 18 mg/kg/day morphine, nalbuphine reliably facilitated ICSS despite its simultaneous precipitation of diarrhea, a common sign of opioid withdrawal. Lastly, the enhanced expression of methadone- and nalbuphine-induced facilitation of ICSS was also observed more than 2 weeks after termination of repeated morphine, a time when signs of morphine dependence and withdrawal had dissipated (figure 4.6).

7.5. Rate-dependency of morphine effect (phase 1)

“Rate-dependency” in behavioral pharmacology describes a phenomenon in which the effect of a drug on the rate of a behavior varies systematically as a function of the baseline, pre-drug rate of that behavior (Dews, 1958; Sanger and Blackman, 1976; Dews, 1981). Rate-dependent effects of a given drug dose under FI schedules are typically manifested as some degree of increase in low rates of behavior coupled with smaller increases or with decreases in higher rates of behavior, and this relationship is often displayed as a negatively sloped line on a graph that plots baseline rate on the abscissa (usually expressed “log baseline rate”) as a function of rate after drug administration on the ordinate (usually expressed as “log % baseline rate”) (Dews, 1964; Kelleher and Morse, 1968; McMillan, 1973; Sanger and Blackman, 1976). We

found previously that abuse liability of monoamine releasers may be related to expression of rate-dependent drug effects in assays of ICSS (Bauer *et al.*, *in press*). A key finding of the present study was that repeated morphine treatment increased the rate dependence of morphine effects on ICSS. Early in the time course after acute administration, morphine effects on ICSS displayed rate dependence only insofar as the highest morphine dose (10mg/kg) decreased high ICSS rates maintained by high frequencies of stimulation more than it decreased lower ICSS rates maintained by lower frequencies of stimulation. This generally agrees with the rate dependence of acute morphine effects in previous studies using other schedules of reinforcement and other consequent stimuli. For example, morphine primarily decreased food-maintained response rates maintained under different schedules of reinforcement in rats, and these effects were rate-dependent insofar as high rates were decreased more than low rates (Thompson *et al.*, 1970). Acute morphine has been reported to increase low response rates maintained during the early segments of fixed-interval schedules or under a differential-reinforcement-of-low-rates- of-responding schedule (McMillan and Morse, 1967; Ford and Balster, 1976), although these rate-increasing effects may be dependent on the consequent stimulus (McKearney, 1980). In chapter 2, low doses of acute morphine also occasionally increased response rates, but these effects were not rate-dependent and were most evident for intermediate ICSS rates maintained by intermediate frequencies of brain stimulation. Morphine exposure associated with longer pretreatment times or with repeated morphine treatment produced qualitative and quantitative changes in the rate dependency of morphine effects, increasing the correlation coefficients and slopes of rate-dependency plots and shifting these plots

vertically upward such that morphine primarily increased low rates of ICSS rather than decreasing high rates of ICSS. In this regard, morphine effects after repeated morphine qualitatively resembled rate-dependent effects of central nervous system stimulants such as amphetamine (Sanger and Blackman, 1976; Do Carmo *et al.*, 2009; Bauer *et al.*, *in press*). To our knowledge, this is the first study to report a change in rate-dependent effects of morphine or any other mu opioid receptor agonist produced by repeated drug treatment. Moreover, insofar as stimulant-like and rate-dependent facilitation of ICSS is predictive of abuse liability, these findings are consistent with the hypothesis that the abuse liability of morphine increases with repeated exposure (Negus *et al.*, 2006; Bauer *et al.*, *in press*).

7.6. Effects of opioids on acid-depressed ICSS (phase 2)

Morphine and other mu agonists are potent analgesics, and they are used for severe and chronic pain conditions. In this project, these drugs were tested in an assay of pain-depressed behaviors in rats that was used previously to study morphine (Pereira Do Carmo *et al.*, 2009), and as in that original study, results were compared to results from an assay of pain-stimulated behavior. Specifically, intraperitoneal injection of 1.8% lactic acid served as an acute noxious visceral stimulus to stimulate a stretching response and depress ICSS. The original hypothesis was that mu opioid agonists, which are effective clinically to treat pain, will block acid-stimulated stretching and acid-induced depression of ICSS.

In this study, all mu opioid agonists produced a dose-dependent decrease in acid-stimulated stretching. The antinociceptive effects observed in this assay were

similar to antinociceptive effects of mu opioid agonists in many other assays of pain-stimulated behavior, and such data have often been interpreted as evidence of analgesic effects of mu agonists. However, exclusive reliance on pain-stimulated behaviors to evaluate effects of opioids or other candidate analgesics is problematic for several reasons (Negus *et al.*, 2006). Most importantly, drug-induced decreases in pain-stimulated behavior can be produced not only by a selective reduction in sensory sensitivity to the noxious stimulus (i.e. true analgesia) but also by nonselective effects such as motor impairment (resulting in “false positive” effects). Insofar as mu agonists are known to function as clinically effective analgesics under a wide variety of conditions, including the treatment of visceral pain (Yuan *et al.*, 2010; Carter and Green, 2011; O'Connor and Rao, 2012), we anticipated that mu agonists would also produce antinociception in assays of pain-depressed behavior.

Accordingly, drugs with variable efficacy at the mu receptor were tested to determine their potency to block acid-induced depression of ICSS. At first, all drugs were tested for their antinociceptive effect as a pretreatment to intraperitoneal injection of 1.8% lactic acid. During this phase, all mu agonists produced dose-dependent blockade of acid-induced depression of ICSS. This is consistent with their efficacy to block acid-stimulated stretching in this study. Also, my findings are consistent with the ability of mu agonists to block many other examples of pain-depressed behavior produced by several other types of pain manipulation, such as pain-depressed feeding, and pain-depressed locomotion (Martin *et al.*, 2004; Neubert *et al.*, 2005; Neubert *et al.*, 2006). These findings suggest considerable generality in the ability of mu agonists to block pain-related depression of behavior.

High-efficacy agonists like methadone produced inverted U shaped dose-effect curves such that peak blockade of acid-induced depression of ICSS was produced by intermediate doses, whereas higher doses produced weaker acid blockade, perhaps because rate-decreasing effects of the high-efficacy agonist limited expression of antinociception. Moreover, high-efficacy agonists were generally more potent to block acid-induced depression of ICSS than acid-induced stimulation of stretching. Conversely, lower efficacy agonists produced only a monotonic and dose-dependent blockade of acid-induced ICSS depression across a broad dose range, and these drugs also tended to be less potent to block acid-induced depression of ICSS than acid-induced stimulation of stretching. These results show the potential for efficacy-dependent mu agonist effects in assays of pain-depressed vs. pain-stimulated behavior, but further studies will be required to assess generality across other mu agonists and to determine underlying mechanisms.

Phase two also included control experiments that examined effects of test drugs administered alone in the absence of noxious stimulation (open bars in figures 5.3; 5.4; 5.5). Except for morphine, all drugs produced mild, but significant, facilitation of ICSS in the absence of lactic acid, suggesting that these drugs may produce non-selective facilitation of ICSS. These results may stand against their “selective” antinociceptive effects after lactic acid administration. However, three findings suggest that mu agonist blockade of acid-induced depression of ICSS was not due to non-selective facilitation of ICSS. First, non-opioid analgesics that are used to reduce pain in humans, such as NSAIDs, were also effective to block acid-induced depression of ICSS (Negus *et al.*, 2012). On the other hand, drugs that fail to produce analgesia in humans, such as

kappa opioid receptor agonists, failed to block acid effects in this assay (Wadenberg, 2003; Negus *et al.*, 2010; Negus *et al.*, 2012). Second, all mu agonists were more efficacious to block acid-induced depression of ICSS than to facilitate ICSS in the absence of acid. For example, 0.1 mg/kg methadone produced a 20% increase in ICSS compared to vehicle when given as a pretreatment to acid vehicle (open bars; figure 5.3). However, when the same dose was administered prior to 1.8% lactic acid, it produced a 38% increase in ICSS compared to vehicle (filled bars; figure 5.3). This suggests that non-selective facilitation is not sufficient to explain blockade of acid-depressed ICSS. Third, the magnitude of opioid-induced facilitation of ICSS in the absence of acid (in these opioid naïve rats) is modest compared to other drugs, such as stimulants. For instance, cocaine failed to block acid-induced depression of ICSS at doses that significantly facilitated ICSS (Negus *et al.*, 2012), and a high dose that did block acid-induced depression of ICSS also produced more than a 50% increase in ICSS in the absence of acid. Mu agonists did not produce the same profile as cocaine, and all of them produced antinociception at doses that did not affect ICSS, or that only mildly facilitated ICSS.

The ability of high-efficacy mu opioid agonists (methadone, fentanyl, and hydrocodone) to facilitate ICSS under control conditions in figures 5.3 and 5.4 was surprising and did not agree with previous findings shown in chapters 3 and 4, where high-efficacy ligands tended to depress ICSS at high doses with little or no evidence of ICSS facilitation at low to intermediate doses. Two factors may contribute to this difference. First, although each drug was tested in a separate group of naïve rats, each drug dose was given twice during phase 2, once prior to acid vehicle, and once prior to

1.8% lactic acid, whereas each drug dose was administered only once during phase 1. As shown in chapter 3, a history of intermittent opioid administration enhances the expression of rate-increasing effects, and subjects in phase 2 received double the injections that subjects in phase 1 were receiving. Second, subjects in phase 2 also received intermittent exposure to a noxious stimulus of 1.8% lactic acid, which might enhance the release of endogenous opioids (Kamata *et al.*, 1986), and this in turn might contribute to the enhancement of rate-increasing effects produced by exogenous mu agonists.

7.7. Manipulation of noxious stimulus intensity (phase 2)

In the previous section, low- and high-efficacy mu opioid agonists were effective to produce antinociception when 1.8% lactic acid was used as the noxious stimulus. Preclinical research with assays of pain-stimulated behavior suggests that low efficacy analgesics may become ineffective to induce antinociception when a high-intensity noxious stimulus is used (Morgan *et al.*, 1999; Negus and Mello, 1999; Cook *et al.*, 2000). Likewise, high efficacy mu agonists are preferred over low-efficacy ligands in severe cases of pain (Prommer and Ficek, 2012). In the present study, using 5.6% lactic acid was intended to increase the intensity of the noxious stimulus.

The failure of 5.6% acid to produce significant stimulation of stretching is consistent with previous findings (Pereira Do Carmo *et al.*, 2009), and this finding can produce greater behavioral depression that reduces not only normally adaptive behaviors such as locomotion, but also pain-stimulated behaviors (Stevenson *et al.*, 2009). On the other hand, lactic acid produced a concentration-dependent decrease in

ICSS, and this is consistent with intensity-dependent effects of other noxious stimuli on other pain-depressed behaviors such as food-maintained operant responding (Neubert *et al.*, 2005). Consequently, it was possible to test opioid antinociception in the assay of acid-depressed ICSS but not in the assay of acid-stimulated stretching when the noxious stimulus was 5.6% lactic acid.

The original hypothesis was that an increase in noxious stimulus intensity would produce a greater decrease in the potency and/or maximal effect of a low-efficacy mu agonist than of a high-efficacy mu agonist. This hypothesis was derived from principles of receptor theory and from previous findings that related drug efficacy at the mu receptor to the intensity of the noxious stimulus (for review; McCormack *et al.*, 1998), although all of these findings relied exclusively on assays of pain-stimulated behavior to test antinociception. Figure 7.1 summarizes this theory of possible outcomes due to an “efficacy X noxious stimulus intensity” interaction.

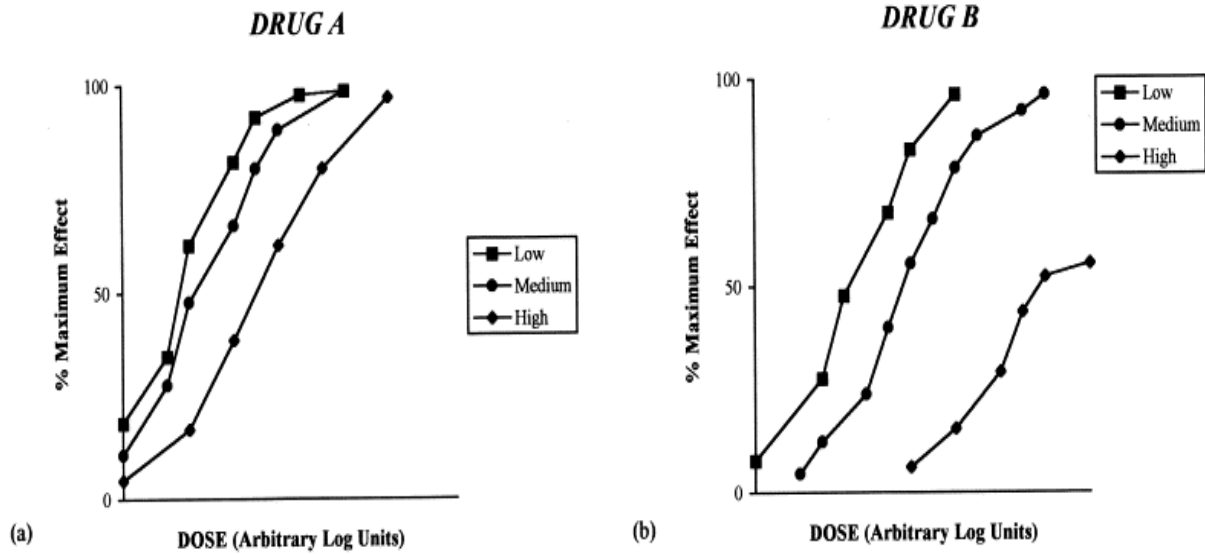


Figure 7.1. Simplified cartoon summarizes the effect of noxious stimulus intensity on two hypothetical drugs that differ in their intrinsic efficacy at the mu receptor. For further clarification, “DRUG A” represents a high-efficacy ligand (e.g. methadone), and “DRUG B” represents a low-efficacy ligand (e.g. nalbuphine). Low, Medium, and High indicate the intensity of the noxious stimulus. Adapted from (McCormack *et al.*, 1998).

In agreement with the hypothesis, the maximally effective methadone dose against 1.8% acid (0.1 mg/kg) lost its antinociceptive effect after increasing the acid concentration, but 1.0 mg/kg methadone was effective. Similarly, the maximally effective nalbuphine dose against 1.8% acid (1.0 mg/kg) also lost its antinociceptive effect at the higher acid concentration, but a higher nalbuphine dose of 10 mg/kg was effective. Overall, the increase in noxious stimulus intensity produced a 10-fold decrease in methadone potency and larger 15-fold decrease in nalbuphine potency without reducing the maximal effect of either drug (table 5.1). Taken together, these results support our hypothesis of greater potency reductions for the low- vs. high-efficacy mu agonist; however, the magnitude of the difference across drugs was modest especially if compared to previous findings with assays of pain-stimulated behavior. For example, Morgan et al. compared antinociceptive effects of morphine and the lower efficacy mu agonist buprenorphine in Lewis rats tested with a warm-water tail-withdrawal assay (Morgan *et al.*, 1999). Both morphine and buprenorphine produced dose-dependent and complete antinociception at a stimulus intensity of 50°C. Increasing the stimulus intensity to 56°C reduced morphine potency by approximately 5-fold, but buprenorphine potency was reduced more than 30-fold, and the maximal effect of buprenorphine was also reduced. Thus, the increase in thermal stimulus intensity in that assay of pain-stimulated behavior resulted in greater dissociation in antinociceptive effects of high- vs. low-efficacy mu agonists than the increase in chemical noxious stimulus intensity in the present assay of pain-depressed behavior.

The reason for the small difference in effects of stimulus intensity on low- vs. high-efficacy mu agonist antinociception is not known. One possibility is that opioid

antinociception in this assay of pain-depressed behavior has a relatively low efficacy requirement, such that relatively large increases in noxious stimulus intensity may be required to reduce maximum effects even of low-efficacy agonists. Consistent with this possibility, mu agonists are generally much more potent in this assay of acid-depressed ICSS (e.g. morphine $ED_{50}=0.124$ mg/kg SC) than in thermal nociceptive assays of pain-stimulated behavior (e.g. morphine $ED_{50}=2.53$ mg/kg IP in Lewis rats tested at $50^{\circ}C$; Morgan et al., 1999). In a related possibility, as mentioned in the section 1.5, lactic acid releases free protons that activate TRPV1 receptors and/or acid sensing ion channels located in the peritoneal region. The expectation is that an increase in acid concentration from 1.8 to 5.6% will activate more receptors, and hence produces more nociceptor activation. However, it is possible that 5.6% acid is not a high enough stimulus to dissociate between high- and low-efficacy opioids in ICSS. As a suggested future experiment, a higher acid concentration or a different noxious stimulus could be used to dissociate between methadone and nalbuphine effects on pain-depressed ICSS. One limitation, however, in using higher acid concentration is the possible lethal outcomes, which limit the number of test sessions conducted for each rat.

In this study, both methadone and nalbuphine produced antinociception after low- and high- acid concentrations. Due to their sedative effects, high-efficacy ligands produce a greater depression in behavior than low-efficacy mu agonists such as locomotion or operant responding. This was observed in this study during phase 1, where methadone but not nalbuphine produced dose-dependent rate-decreasing effects in ICSS in naïve subjects (figure 3.3), and during phase 2 after high doses of methadone, fentanyl, and morphine. In pain-stimulated behaviors, it is possible that

high-efficacy ligands produce antinociception during high-noxious stimulus intensity through “true” analgesia, or through a general decrease in behavior (false positive), or both. The same concept does not apply to assays of pain-depressed behavior, such that increasing the dose of the high-efficacy ligand will recruit some of their rate-decreasing effects, and this may obscure analgesic efficacy.

A suggested experiment to further dissociate between low- and high-efficacy mu agonists in assays of pain-depressed ICSS is by inducing tolerance to the rate-decreasing effects by repeated administration of a mu agonist, such as morphine. As discussed during phase 1, repeated morphine produced tolerance to the rate-decreasing effects of high-efficacy mu agonists. Also, repeated morphine did not produce tolerance to their rate-increasing effects, and in fact, it enhanced expression of rate-increasing effects produced by both low- and high-efficacy mu agonists. Repeated morphine can produce desensitization of mu receptors at different brain areas (Sim *et al.*, 1996). Thus, by decreasing the total number of functional mu opioid receptors in the brain, it is possible to dissociate between the low- and high-efficacy ligands to produce antinociception in assay of pain-depressed ICSS. Consequently, antinociceptive effects of methadone and nalbuphine could be determined in the presence and absence of 1.8% lactic acid in rats that were treated with chronic morphine. Since a) high-efficacy ligands are effective to treat severe pain in the clinic, and b) they are also effective to produce antinociception in assays of pain-stimulated behaviors during high-noxious stimulus intensities in preclinical studies, I predict that methadone will maintain its efficacy to produce antinociception in the assay of acid-depressed ICSS, while the antinociceptive effects of nalbuphine will be more sensitive to repeated morphine

treatment and will not block acid-induced depression of ICSS under the same conditions.

7.8. Morphine antinociception after repeated administration (phase 2)

Chapter 6 tested the antinociceptive effects of morphine in acid-stimulated stretching and acid-depressed ICSS after repeated chronic morphine administration. One possibility for opioid-induced antinociception is that many drugs, including high-efficacy mu agonists, can produce behavioral depressant effects that might augment apparent antinociception in assays of pain-stimulated behavior (in which antinociception is indicated by a depression of the target behavior). On the other hand, behavioral depressant effects could actually oppose and limit expression of antinociception in an assay of pain-depressed behavior (in which antinociception is indicated by increases in the target behavior). During phase 1, repeated morphine produced tolerance to its own rate-decreasing effects. If rate-decreasing effects contribute to morphine-induced antinociception in assays of pain-stimulated behavior, then repeated morphine might also produce tolerance to morphine effects in these assays. Conversely, if rate-decreasing effects do not contribute to morphine antinociception in assays of pain-depressed behavior, then repeated morphine might not produce tolerance to morphine antinociception in these assays; rather, repeated morphine might even enhance antinociception in these assays by producing tolerance to rate-decreasing effects that initially oppose and limit expression of antinociception. Thus, the hypothesis was that morphine would maintain or increase its antinociceptive potency in the assay of acid-induced depression of ICSS, but that tolerance would develop to morphine

antinociception in the assay of acid-stimulated stretching. Results from this study agree with this hypothesis.

The development of tolerance to the antinociceptive effect of morphine in acid-stimulated stretching is consistent with previous reports of opioid antinociceptive tolerance in acid-stimulated stretching and other assays of pain-stimulated behavior (Su *et al.*, 2000; Dong *et al.*, 2006; Trang *et al.*, 2009). On the other hand, morphine antinociception was resistant to tolerance in the assay of acid-depressed ICSS. This is the first evaluation of effects produced by repeated morphine in an assay of pain-depressed behavior. However, these results with morphine are similar to previous findings with the delta agonist SNC80, showing that SNC80 pretreatment produced acute tolerance to the antinociceptive effects of SNC80 in the assay of acid-stimulated stretching but actually enhanced expression of SNC80 antinociception in the assay of acid-depressed ICSS (Negus *et al.*, 2012). These data suggest that morphine antinociception is more vulnerable to tolerance in the assay of acid-stimulated stretching than in the assay of acid-depressed ICSS.

The development of tolerance depends on the drug that is administered chronically, the daily-injection dose and treatment regimen, and the duration of repeated treatment. In this study, escalating doses of morphine were administered chronically for 1 week, and this regimen was sufficient to induce tolerance in acid-stimulated stretching. Repeated morphine administration enhances the expression of abuse-related rate-increasing effects of morphine (Altarifi and Negus, 2011; Altarifi *et al.*, 2012), and this is not likely to account entirely for tolerance-resistance in the assay of acid-depressed ICSS for two reasons. First, the chronic morphine regimen used in the

antinociceptive tolerance study involved the administration of lower morphine doses for a shorter period of time compared to the regimen used in phase one (10 mg/kg/day for 7 days compared to 3.2-18 mg/kg/day for 28 days, respectively), and hence, less enhancement to the rate-increasing effect of morphine compared to phase 1. Second, 1.0 mg/kg morphine was the dose to produce antinociception in both groups, and this dose produced mild facilitation of ICSS in the chronic morphine treated group (16% increase versus acid vehicle). However, the same dose facilitated ICSS by 42% (versus acid alone) when administered prior to 1.8% acid.

It is possible that morphine antinociception in pain-stimulated stretching and acid-depressed ICSS is mediated through distinct mu opioid receptor populations that may respond differentially to chronic morphine. For example, repeated morphine administration produces desensitization (Ferguson, 2001) and downregulation (Kieffer and Evans, 2002) of the mu opioid receptor, and chronic morphine treatment selectively decreased mu agonist-stimulated G-protein activation in brainstem nuclei but not in the forebrain structures (including NA and amygdala) thought to contribute to the stimulant effects of opioids (Sim *et al.*, 1996). Thus, it is possible that morphine-induced antinociception in acid-depressed ICSS is mediated through these desensitization-resistant receptors in the forebrain structures.

The absence of tolerance development to morphine in the assay of acid-depressed ICSS is paralleled by clinical findings indicating that opioids can maintain analgesic efficacy for treatment of chronic and severe pain (Watson, 2012). Tolerance is defined as a decrease in subject's reaction to a specific drug or concentration, that requires an increase in drug concentration to achieve the same desired effect.

Tolerance to a drug effect may develop due to pharmacological reasons (such as receptor desensitization), or pathological reasons (such as disease progression). Many clinical studies showed that subjects who are maintained on relatively constant doses of opioids in healthy individuals (Cooper *et al.*, 2012) as well as in patients who suffer from chronic pain (Cowan *et al.*, 2001) do not show any signs of analgesic tolerance.

Although opioid dose escalation is necessary in some cases to maintain analgesic effectiveness of opioids (Collett, 1998), this loss of analgesia is often related to factors other than pharmacological tolerance, including disease progression (Portenoy, 1994; Portenoy and Savage, 1997).

A final interesting finding in chapter 6 is the enhancement of stretching and exacerbation (although not significant compared to control group) of acid-induced depression of ICSS in the repeated morphine treated group. This is qualitatively similar to hyperalgesia that has been shown to develop in other preclinical assays such as tail flick and paw withdrawal after repeated morphine treatment (Dong *et al.*, 2006; Ross *et al.*, 2012; Wei and Wei, 2012). Also, this repeated morphine-induced hyperalgesia was also reported in clinical settings, where patients receiving opioids for the treatment of pain may actually become more sensitive to pain (De Conno *et al.*, 1991; Chu *et al.*, 2008). In the current study, morphine, lactic acid, or their vehicles were administered 24 hrs after the last daily morphine injection on the previous day. Subjects during this period are possibly in a spontaneous-withdrawal period. Although the direct mechanism of such pain-exacerbation is not exactly known, multiple molecular mechanisms may be involved in such morphine-induced hyperalgesia.

One possibility for this morphine-induced hyperalgesia is the enhancement of TRPV1 channel current during repeated morphine treatment, as shown previously by Ross et al 2012 (Ross *et al.*, 2012). In the same study, they also showed that repeated morphine administration is associated with hyperexcitability and functional remodeling of sodium channels in sensory neurons. Another possibility is that repeated morphine treatment may activate the descending pain facilitation arising in the RVM (Vanderah *et al.*, 2001). A final possibility is an alteration in opioid receptor signaling after chronic opioid administration, such that there is a shift in opioid receptor G-protein signaling from predominantly Gi/o inhibitory to Gs stimulatory following chronic in vivo morphine exposure (Crain and Shen, 2000; Gintzler and Chakrabarti, 2000).

7.9. Conclusions and future directions

The main purpose of the above studies was to validate intracranial self-stimulation as an assay of pain-depressed behavior in preclinical research. The goal was to find a novel animal model to measure pain-related changes in behavior in preclinical research, which may generate better predictive validity outcomes to the clinic. Morphine and other opioids were tested in these studies due to their known analgesic efficacy in the clinic. Opioid pharmacology was studied in ICSS, and in an assay of pain-stimulated behavior. The author predicts that assays of pain-depressed behavior in animal research may provide a better “translational” tool to examine analgesic properties of candidate drugs in animals. A good analgesic would block pain in both assays, and may also be less susceptible to analgesic tolerance after chronic administration. By using these assays of pain-stimulated and pain-depressed behavior

and comparing effects of novel drugs to effects of opioid analgesics, it may be possible to identify new candidate analgesics that have less abuse-potential than opioids (i.e. do not facilitate ICSS).

One limitation for using ICSS as a behavioral baseline in assays of pain-depressed behavior is its sensitivity to stimulant effects of test drugs. Although this assay has been useful to test abuse-related stimulant effects of new drugs, drugs that produce non-selective facilitation of ICSS in the absence and presence of noxious stimulus may fail to produce analgesia in humans. Thus, one suggested future experiment is to investigate other behavioral baselines that may be less susceptible to false-positive results, such as pain-depressed feeding and pain-depressed social interaction. Another possible future direction is to test different types of pain modalities that would be more clinically relevant, such as cancer pain and chronic pain.

Finally, a good strategy to enhance pain management is either to find alternative analgesics to the ones that are currently used in the clinic, or to minimize the disadvantages of currently used analgesics. Opioids are widely used in the clinic, but they also have a high abuse potential. The above studies enhanced our knowledge of some of the factors that could possibly be involved in opioid addiction. Future studies could build on this information by seeking strategies to retain analgesic effects of mu agonists while reducing abuse liability. For example, delta-opioid agonists (such as SNC80) showed promising results to produce antinociceptive effects in assay of pain-depressed behavior with less abuse-liability compared to mu agonists. These drugs could potentially replace or minimize the dose required of mu opioid agonist to produce clinical analgesia.

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Abstracts

Altarifi AA, Negus SS (2012). Effects of Mu Opioid Receptor Agonists on Pain-Stimulated and Pain-Depressed Behaviors in Rats. Poster presented at Society of Neuroscience, New Orleans, Louisiana 2012

Altarifi AA, Negus SS (2012). Effects of Mu, Delta and Kappa Opioid Receptor Agonists on Pain-Stimulated vs. Pain-Depressed Behaviors in Rats. Poster presented at International Narcotics Research Conference; Kansas City, Missouri 2012

Altarifi AA Negus SS (2012): Effects of Mu, Delta and Kappa Opioid Receptor Agonists on Pain-Stimulated vs. Pain-Depressed Behaviors in Rats. 7th annual pain consortium symposium: NIH, (May 2012)

Altarifi AA, Negus SS (2011). Chronic Morphine Treatment Influences Mu- Opioid Receptor Agonist Effects on Intracranial Self-Stimulation. Poster presented at the College on Problems of Drug Dependence: Hollywood, Florida, 2011

Altarifi AA, Negus SS (2011). Effects of Mu Opioid Agonists on Intracranial Self-Stimulation in Rats: Role of Mu Agonist Efficacy. Poster presented at Experimental Biology: Washington, D.C., 2011

Altarifi AA, Negus SS (2010). Role of Time Course and Repeated Treatment as Determinants of Morphine Effects on Intracranial Self-Stimulation (ICSS) in Rats. Poster presented at Experimental Biology: Anaheim, California, 2010

Presentations

(2012): Effects of Mu- Opioid Receptor Agonists on Pain-Depressed Behaviors in Rats. Presentation at the Emory/Wake Forest/VCU lab exchange, Winston-Salem, NC (Sep, 2012)

(2011): Mu but not kappa opioid receptor agonists block pain-depressed behavior of intracranial self-stimulation in rats. International Narcotics Research Conference: Hollywood, FL, (June, 2011)

(2010): Effects of Mu Opioid Agonists on Intracranial Self-Stimulation in Rats: Role of Mu Agonist Efficacy. Presentation at the Emory/Wake Forest/VCU lab exchange, Winston-Salem, NC (Oct, 2010)

(2009): Effects of Acute and Chronic Morphine on Intracranial Self-Stimulation in Rats. Presentation at the Emory/Wake Forest lab exchange: Atlanta, GA (Sep, 2009)

Professional Memberships

College on Problems of Drug Dependence (CPDD)

American Society of Pharmacology and Experimental Therapeutics (ASPET)

Central Virginia Chapter of the Society for Neuroscience (CVCSN)

Society of Neuroscience (SFN)

International Narcotics Research Conference (INRC)

Other Training and Experience

2011-present Student Government Association (SGA) representative of the Pharmacology and Toxicology Student Organization (PTSO)

March, 2013 *Community Outreach*: screening and judging at the “Richmond Metro Science fair” (=Metro Richmond STEM Fair).

- 2012-2013 Graduate Student Representative of the Central Virginia Chapter of the Society of Neuroscience (CVCSN). (*Elected*)
- May, 2012 *Community Outreach:* Lecturing to AP psychology; Hermitage High School, Richmond, VA
- 2006-2008 *Research and Teaching Assistant:* Department of Anatomy and Neurobiology-Jordan University of Science and Technology (Irbid, Jordan)
- 2005- 2006 *General Dental Practitioner:* Zarqa-Jordan
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Awards

- 2012 *Travel Award:* Central Virginia Chapter Society for Neuroscience (CVCSN); Society of Neuroscience (SFN), New Orleans, Louisiana (2012)
- 2012 *Travel Award:* International Narcotics Research Conference (INRC); Kansas City, Missouri (2012)
- 2011 *Travel Award:* The American Society of Pharmacology and Experimental Therapeutics (ASPET), Experimental Biology, Washington, D.C. (2011)
- 2003 *Tuition Waiver:* Ranked 1st among 2004 dental class in Jordan University of Science and Technology (Irbid, Jordan)