EXAMINING THE IMPACT OF OPIOID WITHDRAWAL ON PAIN PROCESSING: THE INFLUENCE OF SOCIAL ISOLATION STRESS

by

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ABSTRACT

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The use of opioids as a traditional treatment for acute and chronic pain has been severely hindered by the addictive nature of these substances and the pain relief they provide. Clinical reports suggest that opioid addicts are hypersensitive to pain during abstinence, and this effect may persist for months afterward. Examinations of nociceptive processing during opioid withdrawal in rodents have produced mixed results, with little evidence of decreased thresholds or latencies to noxious stimuli. To date, no studies have explicity evaluated pain affect during the withdrawal period. Therefore, the purpose of the current study was evaluate both sensory and affective pain processing in response to opioid withdrawal as well as the impact of social isolation stress on these measures. Sensory pain processing was examined during the seven-day morphine dosing period and over a five day period following abstinence. Pain affect was evaluated during the withdrawal period, following the induction of an experimental inflammatory condition. The doses of morphine selected produced robust analgesia and a reliable withdrawal syndrome. The results demonstrated no changes in sensory pain processing in response to morphine or social isolation during the withdrawal period, but differential effects of morphine and social isolation on pain affect on the first and second days of withdrawal. Group-housed subjects in morphine withdrawal demonstrated increased pain affect relative to saline-dosed subjects, but only on the

first day of testing. Socially isolated subjects demonstrated decreased pain affect in comparison to group-housed subjects on the first and second day only, and no difference between socially isolated morphine- and saline-dosed subjects were present. The current study provides evidence of altered emotional pain processing during withdrawal, which could contribute to the development of novel treatments for opioid addicts with underlying chronic pain conditions.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

Table Page

CHAPTER 1

INTRODUCTION

The use of pharmaceutical intervention has been a pillar in the medical community for centuries, starting with the use of medicinal herbs and progressing to modern synthetic drugs for a multitude of diseases and conditions. Although the benefits of pharmaceuticals are significant, there are often undesired side effects that can complicate treatment. The development of physical and psychological dependence on a substance is one of these concerns. Medicines used to treat chronic pain conditions as well as pain secondary to surgery or injury can be potently analgesic but also carry a serious risk of addiction. Strict controls over the sale and use of addictive substances have made it more difficult to obtain large amounts of these substances, but there are still means of obtaining these items illegally. There is a growing problem with individuals obtaining multiple prescriptions of legal opioids to feed addiction, as well as the presence of illegal drugs such as heroin, with an estimated 1.7 million individuals addicted to legal substances and 282,000 addicted to heroin (SAMHSA, 2008; Comer et al., 2008). In Minneapolis/St. Paul, MN, the number of people entering treatment programs for opiates (including heroin) doubled in the last decade, continuing an upward trend in opioid abuse despite corresponding drops in methamphetamine and cocaine addiction indicators (Falkowski, 2009). Prescription drug abuse has become more popular among adolescents than heroin, methamphetamine, and cocaine combined, and among illicit drug use is second in popularity only to marijuana (Johnston et al., 2010). Research into the genetic and lifestyle risk factors for the development of an addiction as well as those that affect the progress of recovery is vital for the prevention and treatment of these types of addictions. One aspect of this research is the withdrawal syndrome that often accompanies abstinence from a drug of abuse.

1.1 Models of Substance Abuse and Dependence

There are several theories to explain the cycle of substance abuse, abstinence/withdrawal, and relapse that occurs in drug addiction. Drug addiction, like many aspects of individual behavior, is shaped by the same principles that control learning and memory and is heavily influenced by reward and reinforcement. A combination of genetic, environmental and physiological factors influence whether the rewarding properties of drug propagate continued use. While some non-addicted users are capable of indulging without needing continuous re-administration, some individuals easily fall into a pattern of substance abuse. Models of addiction attempt to incorporate these varied factors to explain how occasional, non-problem drug use can lead into the cycle of addiction.

The physical dependence model of addiction states that repeated use of a substance eventually leads to physical dependence, and that any attempts to abstain are quashed by severe withdrawal symptoms, which create a powerful motivation to re-administer the drug and eventually lead to relapse (Jellinek, 1968). Certain triggers may lead to relapse months or years after the last administration when an individual is exposed to conditioned stimuli that are associated with the drug—an old drug dealer's apartment complex, or bar that was frequented during the addiction, for example—and thus an addict is never truly free of his or her condition (Wikler, 1980). The use of methadone, buprenorphine and other opioid agonists to assist recovered addicts is based on this principle—instead of rapidly detoxifying the body of opiates, these substances act as a safer alternative to heroin that can be monitored and slowly decreased in dose until the patient achieves abstinence without severe withdrawal symptoms (Farrell et al., 1994). By administering these alternatives through out-patients programs, an addict can reduce the level of craving associated with daily activities over time, instead of being forced to avoid any stimuli associated with drug use after in-patient rehabilitation.

In contrast, the positive reinforcement model of addiction asserts that the motivation to re-administer drugs of abuse stems from the inherently reinforcing properties of the drug itself,

not the negative impact of abstinence (Charney & Nestler, 2008). The feelings of euphoria, relaxation, or other mood enhancement are naturally rewarding, in the same way as many other stimuli, including food, drink, and sex. The craving for the drug being abused becomes a compulsion, to the point that an individual becomes obsessed with attaining the previous state of euphoria. This model explains why many drug addicts continue to abuse in spite of deleterious health problems, financial ruin, or legal troubles—they simply cannot overcome the compulsion to return to the temporary state of bliss that follows drug administration. The nearly instantaneous reward produced by many drugs overwhelms any potential reward from remaining sober, such as future career goals. The major issue with this model of addiction is that for many long term users there is no longer a distinctive 'high' associated with the drug—they are merely trying to maintain normal functioning which becomes disrupted during abstinence. In addition, there is no evidence from animal studies of self administration that indicates any relationship between the degree of drug effect and the level of craving (i.e., craving increases after repeated use more than during the first few doses, which should be the most rewarding).

The incentive-sensitization/opponent process model takes into account the phenomenon of decreased euphoria in substance abusers as well as the compulsive desire to continue drug use, which essentially combines the physical dependence and positive reinforcement models (Robinson & Berridge, 1993). In this model, repeated administration leads to an increased sensitization for drug craving despite a decrease in drug effects, which decrease over time as the body adapts to continued presence of the substance in the bloodstream. This occurs both in the liver, where enzyme induction helps to break down the substance more readily, and in the brain, where neuronal changes take place to decrease the psychological effects of the drug. Even without the euphoria, any attempt at abstinence intensifies the compulsive desire to re-administer the drug, and relapse occurs. The opponent process model attempts to explain these adaptive changes even further by distinguishing

between the rewarding, mood-elevating properties of the drug (a process) and the delayed, negative affect produced during the comedown or withdrawal (b process). Initial drug use is associated with a strong euphoric effect, invoking changes in mood, decreased anxiety, or other positive effects but the delayed negative effects are negligible. However, repeated use leads to a decrease in the positive mood changes but intensifies the negative affect produced by abstinence. Thus, the user continues to take the drug not to experience the euphoric 'high', but to counter the negative affect of withdrawal and maintain a normal level of affect—according to Koob and Le Moal (1997), this individual experiences dysphoria when abstaining and needs the drug to reach an altered hedonic set point.

The most widely accepted comprehensive model is based on the various biopsychosocial factors that contribute to initial drug use, the transition to substance abuse, and finally cycles of addictive behavior (Donovan & Marlatt, 1988). Proponents of this model take into consideration not only the rewarding aspects of drug effects, but also the psychological and social conditions that precede initial use, including social acceptability, peer influences, and various mood and anxiety disorders. This model is particularly useful when examining the patterns of addictive behavior in chronic pain patients, as the same factors also predict outcomes in the chronic pain population (Stanos, 2007; Gatchel et al., 2007).

While none of these models perfectly describe the addiction cycle for all individuals, they provide a helpful guide for understanding some of the fundamental reasons that an addicted person cannot escape their addiction following a period of abstinence. Some individuals continue to use because the euphoria produced by administration is such a powerful reward that any and all consequences are ignored, others are driven by a desire to avoid withdrawal symptoms, which can be devastating. Finally, many individuals seem to self-medicate with substance abuse, and continue drug use to relieve anxiety, depression, or improve functionality.

1.2 Pharmacological Basis of Withdrawal

The withdrawal syndrome plays an important role in the motivation to continue drug use and is a key aspect of the cycle of addiction in some models of abuse. Withdrawal refers to the physiological processes and psychological cravings that cause an addicted individual to desire more of a substance following a period of abstinence from the drug. The DSM IV-TR lists diagnostic criteria for withdrawal from alcohol, amphetamines, cocaine, nicotine, opioids, sedatives, hypnotics and anxiolytics (2000). The time course of symptom onset varies based on the route of administration and specific characteristics of the drug, but initial signs tend to begins after a few half-lives have passed since the last administration (Fishbain, 2002). Use of drugs through intravenous (IV) injection or inhalation tends to produce a rapid, intense response and withdrawal can appear within hours, while drugs used orally (especially those with lengthy half-lives) may not produce withdrawal symptoms until a few days after the last dose. Signs of withdrawal include physiological and behavioral changes which tend to be the opposite of the normal response to drug administration. These tend to be unpleasant and result in severe negative affect, which compels an addict to administer another dose of drug. Opioids produce sedation when administered, but withdrawal results in behavioral agitation (Martin et al., 1963). Symptoms can be extremely unpleasant, and range from nausea and vomiting to hallucinations, seizures and severe cognitive impairment (DSM IV-TR, 2000).

The presence of a drug in the body for an extended period induces physiological changes designed to counter the drug's effects as well as adapt to the drug’s activities in the body. This leads to the development of tolerance to many of the drug's effects, so that administration of the drug after many encounters fails to produce the same strength of response. Evidence for the formation of tolerance to the hyperthermic effects of opioids was shown in rats, where animals dosed with opioids for several days maintained a normal temperature following opioid administration, while control animals demonstrated a significant increase in body temperature (Martin et al., 1963). The adaptive drop in body temperature

following morphine injection can also be conditioned to a specific environment; the administration of the drug in the presence of a new environment results in the expected increase in body temperature in rats repeatedly dosed with morphine in a different room (Siegel, 1975). These compensatory processes persist when an individual abstains, and produce the characteristic signs of withdrawal representing a sudden disturbance in homeostasis (Himmelsbach, 1943; Sharma et al., 1975).

1.3 Examining the Withdrawal Syndrome in Opioid Dependence: Evidence from Preclinical Models and Clinical Experience

Both human and animal studies have examined the factors that influence tolerance and withdrawal to opioids. Electrophysiology and pharmacology studies have shown that withdrawal symptoms result from hyperactivity in cholinergic and excitatory amino acid pathways, mediated in part by increased activity of glutamate receptors, including NMDA, AMPA and mGlu5 (Wang et al., 1995; Rasmussen & Vandergriff, 2003; Rasmussen et al., 2004). While opioids inhibit activity in the locus coeruleus (LC), withdrawal results in increased output and leads to increased activation in areas that receive projections from this nucleus, including the cerebral cortex, limbic system, cerebellum, and thalamic nuclei (Nestler et al., 1994; Aghajanian et al., 1994; Kimes et al., 1998). The production of cAMP also increases rapidly during withdrawal, and the increased pain signaling during this period has been partly attributed to this increase (Sharma et al., 1975; Bie et al., 2005).

There are behavioral studies that examine factors which alter the pattern and severity of the withdrawal syndrome. In rats, opioid withdrawal produces a stereotypical cluster of behaviors and symptoms, including diarrhea, wet dog shakes, increased production of tears and mucus, hypothermia, decreased sleep, and hostility (Martin et al., 1963). Several of these signs or behaviors can be quantified to produce an overall score indicating the severity of the

withdrawal syndrome. In humans, subjective reports (self-report or observed) can be used to quantify the level of unpleasantness experienced during withdrawal (Handelsman et al., 1987). Manipulating certain factors can lead to reduced withdrawal scores, while others increase the severity of symptoms. Heroin addicts report a sharp increase in withdrawal severity only days from the last IV dose, which lasts for about a week before subsiding. This is strikingly different from the pattern seen when abstaining from oral methadone or buprenorphine after diminished dosing: withdrawal symptoms peak after several days at a much lower level of severity and persist at low levels for a few weeks (see Farrell et al., 1994 for review). Unfortunately, rapid detoxification from high doses of methadone is reportedly more severe than sudden heroin withdrawal; incarcerated prisoners who had been forced to abstain following arrest described seeing methadone patients suffer for weeks with severe symptoms, while heroin users were only experienced symptoms for the first week of abstinence (Mitchell et al., 2009). An additional concern for recovering addicts using opioid agonists to wean themselves from opiates is that replacing heroin with methadone or L-alpha-acetylmethadol (LAAM) simply exchanges one addictive substance for another. Pharmacological studies have demonstrated methadone to be as potentially addictive as morphine, and many patients complain of cravings between daily doses (Dyer & White, 1997). On the other hand, carefully monitored oral dosing of opioid agonists can be very effective in overcoming dependence, and is much safer than the use of IV heroin purchased from street dealers (Simpson et al., 1982; Bertschy, 1995). In addition, the use of opioid antagonists, such as naltrexone, can be effective in reducing physical dependence; however, the patients who are prescribed these substances will still experience craving and must have sufficient motivation and support to avoid relapse (O'Brien, 1993).

The use of animal models enables researchers to examine the underlying physiological changes that lead to opioid tolerance and withdrawal. Rodent studies model opioid dependence through repeated daily injections of various opioids (usually morphine), the use of subcutaneous pellets of drug, or by surgically implanting an infusion pump underneath the skin to deliver

opiates either systemically or into specific areas of the central nervous system. The development of opioid dependence and the time course of withdrawal is highly dependent on the route of administration and dosing regimen. Withdrawal symptoms appear within a day of the last injection in a regimen, or can be precipitated by the administration of antagonists (naltrexone or naloxone) to block the mu and delta opioid receptors. The latter method ensures that all subjects experience the abstinence syndrome on a similar time scale, and also eliminates the need to wait up to 24 hours before symptoms appear (Blasig et al., 1973; Resnick et al., 1977). However, the symptoms produced in response to naltrexone are short lived and do not allow for extended assessment of withdrawal-induced changes (Bodnar & Kest, 2009). On the other hand, this method of precipitated withdrawal is preferable for use in human-subjects studies in patients who are being maintained on opioid antagonists, to reduce the probability of relapse during the experiment.

Studies comparing the effects of repeated injections to implanted pellets have found that pellets produce high, stable circulating levels of drug, while injections induced short-lived peaks in blood levels (Fischer et al., 2008). Pellets produced dependence more reliably and were able to induce changes in the proliferation of cells of the dentate gyrus that were not seen following repeated injections. Despite these advantages, the presence of constant high levels of opiates in the bloodstream would not be feasible for most opioid addicts outside of a clinical setting with an IV, and so the use of repeated doses is able to better model the peaks and valleys experienced by a user on a given day. Unfortunately, the use of any form of injections that involves restraint and discomfort will be stressful to the subject, and self-administration paradigms cannot ensure that all subjects receive the same doses at the same time. An ideal system would utilize an infusion pump with time-release functions, to boost blood levels at certain intervals during the day (say, 4-6 hours) instead of constantly infusing high levels of the drug.

Pharmacological studies in rodents have demonstrated that morphine tolerance and withdrawal symptoms can be attenuated through the co-administration of several drugs, including NMDA glutamate receptor antagonists (MK-801 and ketamine), as well as nitric oxide synthetase inhibitors (L-NAME), and opioid antagonists such as naltrexone (Inturrisi, 1997). However, drugs that act on the glutamate receptors fail to attenuate withdrawal-induced activity in the LC or elevated norepinephrine levels in the limbic system (Rasmussen et al., 1991), and a similar pattern emerges when orexin receptor agonists are used (Sharf et al., 2008). Clinical reports have also suggested that alpha-2 receptor agonists are useful for relieving withdrawal symptoms, and microinjection studies in rodents reported a decrease in behavioral symptoms when clonidine was microinjected into the LC (Taylor et al., 1988). The effects of noradrenergic activation can also be detected peripherally. A study conducted on patients dependent on buprenorphine demonstrated that the level of withdrawal symptoms was positively correlated with plasma concentrations of 3-methoxy-4-hydroxyphenylglycol (MHPG), a metabolite of norepinephrine, indicating that the behavioral agitation produced by opioid withdrawal involves activation of the noradrenergic system (Redmond & Krystal, 1984).

Several studies have examined the influence of specific opioid receptor types on the development of opioid addiction and withdrawal. While the mu-opioid receptor is known to be primarily involved in the analgesia produced by morphine and heroin (Klein et al., 2009), delta and kappa receptors as well as their ligands and several gene products modified by opioid use have contributions of their own (see Le Merrer et al., 2009 for review). Kappa opioid receptors and their endogenous ligand dynorphin play a role in addiction to cocaine and other stimulants, and may be responsible for the some of the negative aspects of dependence, such as craving (Shippenberg et al., 2007; Bruijnzeel, 2009). Kappa opioid receptors are involved in producing the negative affect that leads to conditioned place aversion as well as re-instatement of cocaine-induced place preference following stress exposure (Land et al., 2009). Genetic knockout of the prodynorphin gene or the administration of kappa receptor antagonists

enhances the behavioral sensitization following repeated opioid dosing and the magnitude of the response to naloxone-precipitated withdrawal, while kappa receptor agonists reduce these effects (Shin et al., 2009). Knockout of the gene KEPI, which is located near the gene for the mu receptor and encodes a PKC-enhanced phosphatase 1 inhibitor that is selectively modified by opioid administration (Liu et al., 2002), enhances the development of tolerance to repeated morphine dosing but decreases the rewarding properties of this drug (Drgonova et al., 2009).

There is also evidence that other neurotransmitter systems contribute to opioid tolerance and withdrawal, including glutamate and acetylcholine. The use of receptor antagonists and knockout mice has demonstrated that kainite receptors, particularly those containing subunit GluR5, are involved in the development of opioid reward and tolerance but not the analgesic aspects of these substances (Kest et al., 1997; Carlezon et al., 1999; Bogulavsky et al., 2009). In addition, genetic knockout of the M5 muscarinic receptor did not alter morphine analgesia or tolerance, but eliminated the rewarding aspects of the drug and attenuated the withdrawal syndrome (Basile et al., 2002).

In addition to examinations of pharmacokinetic, genetic, and other modifications of opioid withdrawal, there is also a body of literature that focuses on the risk factors and differences in response based on age, gender, and other conditions. In Eastern India, opioid addicts reported faster onset of withdrawal and tended to have higher income but less education that those addicted to other substances, which emphasizes the role of socioeconomic factors in the risk for opioid addiction (Saddichha et al., 2007). Adolescent mice demonstrated fewer depressive symptoms following opioid withdrawal compared to adult mice, which suggests that age and state of development has an impact on the body's response to opioid dependence (Hodgson et al., 2009). There is also evidence of distinct sex differences in the response to opioid administration (see Bodnar & Kest, 2009 for review). Male rats develop tolerance to morphine more rapidly and to a greater extent than females, though controlling for estrous cycle can decrease this difference (Craft et al., 1999; Shekunova & Bespalov, 2004).

Female rats also demonstrate fewer behavioral signs of withdrawal during morphine abstinence (Craft et al., 1999).

Although there is a great deal of interest in the underlying physiological changes that lead to withdrawal behaviors and craving, little research has focused on the changes in pain processing that are induced by chronic opioid treatment; specifically, what occurs during the withdrawal period and beyond. Areas of the brain involved in processing pain interact with stress, reward, cognitive, and emotional processing, so that the response to noxious stimulation can be modified by internal and external factors, including perceived reward or punishment, attention and memory states, mood, expectation, and environmental stressors. The current model of the pain processing system takes these factors into account by conceptualizing different dimensions of the pain experience: sensory/discriminative, emotional/motivational, and cognitive/attentional mechanisms (Melzack & Casey, 1968). Modification of one or more of these processes alters the subjective pain experience. Sensory pain processing occurs in the lateral pain system, which includes projections from the spinothalamic tract that ascend to the lateral thalamic nuclei, which in turn send projections to the primary somatosensory cortex. Regions along this pathway are important for the perception of location, intensity, and other discriminatory characteristics of a noxious stimulus, and damage to one or more areas produces paraesthesia and/or deficits in the ability to detect and locate noxious sensations from the skin. Affective and motivational processing of nociceptive inputs occurs in the medial pain system, which receives input from the spinal cord via the medial thalamic nuclei and includes limbic structures in the midbrain and neocortical regions such as the anterior cingulate cortex (ACC) and insular cortex. Projections from the medial reticular formation to the midbrain limbic area allow reciprocal communication to occur between the hypothalamus and the midbrain as well as between the medial forebrain bundle and limbic forebrain areas. The medial forebrain bundle is associated with reward processing through its connections to both the ventral tegmental area and nucleus accumbens (Koob, 1992). Together, these limbic regions process

the motivational aspects of stimuli, including the aversive component of pain. Damage to areas involved in the medial system, such as the ACC, disrupts the processing of the emotional component of noxious stimuli while discriminatory capabilities remain intact (LaGraize et al., 2004b). In contrast, stimulation of the mesencephalic regions produces an aversive state normally associated with noxious stimulation, while stimulation of the medial thalamic nuclei results in fear-mediated freezing or escape behavior (Roberts, 1962; Mongeau et al., 2003). The limbic forebrain regions, including the amygdala and hippocampus, process information related to learning and memory for aversive states, including fear and pain. In addition to the limbic system, projections to the neocortex, especially the frontal cortex, are important for the cognitive/attentional mechanisms of pain processing. The conscious experience of pain is influenced by information related to mood states, anxiety, and attention, such as the anticipation of noxious stimulation, placebo effects, traumatic experiences, early life adversity, meditation, and much more. Thus, an individual can volunteer for an extended session of needle injections at a tattoo parlor with little hesitation but experience excruciating pain from a single vaccine needle at a doctor's office.

The interaction between the hypothalamus-pituitary-adrenal axis and the pain processing regions of the brain mediates the influence of stress on pain processing. Dysregulation of corticotropin releasing hormone (CRH) is associated with substance abuse as well as mood disorders, and the use of CRH receptor antagonists as an experimental treatment for depression has demonstrated some success (Zobel et al., 2000). Genetic variations in the genes coding for the mu opioid receptor (OPRM1) and pro-opiomelanocortin (POMC), which is produced in the hypothalamus and is cleaved into adrenocorticotropic hormone as well as beta-endorphin, are associated with altered stress reactivity and increased risk of substance abuse (Bond et al., 1998; LaForge et al., 2000; Bart et al., 2004 & 2005; Kreek et al., 2005). Environmental stressors increase the rewarding aspects of self-administered drugs of abuse in rodents throughout the addiction cycle, and even reinstatement following extinction is enhanced

following stress (Kreek et al., 2005). Recovering cocaine addicts demonstrated increased cardiovascular activity, craving, anxiety, and stress hormones when exposed to stressful or drug-related laboratory conditions but not in response to neutral conditions (Sinha et al., 2003).

The involvement of the HPA axis in mediating distress during withdrawal is of particular importance because the severe negative affect and altered pain processing experienced during withdrawal can increase the risk of relapse in humans. Manipulation of the stress response system can be achieved in the laboratory through early life stress and post-traumatic stress procedures. These procedures involve stressing pups before or after birth or the use of uncontrollable shock during adulthood to enhance stress reactivity and anxiety in laboratory animals, traits which are present in humans that experience childhood abuse, neglect, or trauma (see Fumagalli et al., 2007 for review). Maternal separation in infancy is associated with altered responses to morphine analgesia and mu opioid receptor expression as well as enhanced emotional responses to noxious stimuli (Weaver et al., 2007; Uhelski & Fuchs, 2010).

To date, no studies have examined the effect of early life stress on the response to opioid withdrawal. The few studies that have examined nociceptive processing changes during the withdrawal period in rodents have focused on reflexive behaviors, such as the tail-flick response, that may or may not be altered by repeated opioid administration (Zissen et al., 2007; Sweitzer et al., 2004; Pinelli & Trivulzio, 1997). The clinical data that is available suggests that opioid addicts are hypersensitive to pain while they are still using and continue to demonstrate elevated pain scores during withdrawal and after a period of abstinence (Compton, 1994; Compton et al., 2000; Pud et al., 2006). The use of opioids chronically can lead to changes in the neural pathways that process and modify pain perception, causing increased sensitivity to painful stimuli and increased negative affect (Pud et al., 2006). In recovering addicts, evidence of these changes corresponded with increased drug craving (Redila & Chavkin, 2009).

The purpose of this study was to examine changes in pain processing that occur during the withdrawal period following a chronic opioid regimen. We also evaluated the impact of social

isolation stress on these behaviors. This study provides a new perspective in animal research models of addiction by explicitly evaluating both the sensory aspect of pain processing as well as the emotional response to noxious stimulation. The latter is less common in the animal literature, but the behavioral tests that have been developed to assess pain affect have provided unique information about the response to analgesic drugs, including anti-depressants, and has aided in identifying areas of the brain that are intimately involved in processing the emotional response to pain in both humans and animals (LaBuda & Fuchs, 2000a, 2000b, 2001; LaGraize et al., 2004b).

Specifically, we planned to:

- Examine the behavioral response to mechanical and thermal stimuli in order to determine sensory threshold levels before, during, and after chronic opioid treatment
- Explore the impact of the withdrawal syndrome on pain affect as shown by the induction of inflammation during this period followed by testing in the place/escape avoidance paradigm.
- Determine the impact of social isolation stress on the response to chronic opioid withdrawal as shown by the response to place/escape avoidance testing.

For animals receiving chronic morphine injections, thresholds were expected to decrease significantly during the withdrawal period relative to baseline and the morphine dosing period and remain low throughout withdrawal testing, especially for animals receiving an injection of carrageenan, an inflammatory agent, prior to place/escape avoidance testing. This pattern was also anticipated for thermal threshold scores. It was also hypothesized that animals receiving repeated morphine injections would also demonstrate significantly elevated pain affect, as shown by increased avoidance of noxious stimulation during the place escape/avoidance paradigm (PEAP) tests. Finally, it was hypothesized that stress induced by

social isolation during the juvenile period would also result in increased pain affect that would enhance the effect of the withdrawal syndrome.

CHAPTER 2

METHOD

2.1 Subjects

A total of 77 subjects were utilized for this study. Male Sprague-Dawley rats, between 250 and 350 g at the start of the experimental protocol were used as subjects. An in-house breeding colony maintained in the University's Animal Care Facility provided these animals, and approval was obtained from the University of Texas at Arlington Institutional Animal Care and Use Committee. All animals were treated in accordance with the guidelines set forth by the International Association for the Study of Pain (Zimmerman, 1983). All behavioral tests were conducted by experimenters who were blind to Drug conditions, but not to Housing or Pain condition (inflammatory pain models induce visible swelling and redness, while saline does not). Upon completion of the experimental protocol, animals were euthanized by inhalation of carbon dioxide (CO_2) as recommended by NIH guidelines (Danneman et al., 1997; NIH, 2001).

2.2 Materials and Procedure

Morphine sulfate

An opioid agonist that binds primarily to mu-opioid receptors (similar to endorphins) located throughout the brain and spinal cord. Considered the gold standard in analgesics; novel pharmaceuticals are compared to morphine in drug trials. Metabolized primarily in the liver and reaches peak levels in the blood around 20 minutes after subcutaneous injection (Trescot et al., 2008; Chou et al., 2009).

Carrageenan

A thickening agent derived from red seaweed that is used commonly as a food additive. When injected under the skin, it induces a localized immune response and is used as an animal model of arthritis (Winter et al., 1962).

Behavioral Testing

Sensory Processing

Two behavioral tests were used to assess sensory processing: mechanical paw withdrawal thresholds (MPWT) and the Hargreaves test of thermal nociception. For MPWT, animals were habituated to the mechanical threshold testing chamber for ten minutes. Paw withdrawal thresholds were evaluated using eight von Frey monofilaments, ranging from 2 g to 26 g, in up-down method described by Dixon (1980). Subjects were placed in a Plexiglas chamber above a raised mesh floor (20 x 10.5 x 40.5 cm), which allows for free movement of the animal while still having access to the hind paws for testing. After a ten minute habituation period, the animal's hind paws were stimulated with the lowest von Frey, which was pressed against the plantar skin until the filament bends slightly, and held for one second. If the animal did not respond (by rapidly flinching and/or licking the paw), the next highest force was tested after at least a ten-second interval. When a response was recorded, the paw was tested with the next lowest von Frey, and so on, until a response is not longer generated. This pattern continued until four additional stimuli had followed the original response, or until the highest von Frey was reached without a response. In the latter case, the animal was recorded as having the maximum response for the specific set of von Frey monofilaments. Withdrawal thresholds were calculated using the following formula: [Xth]log = [vFr]log + ky where [vFr] is the force of the last von Frey used, k = 0.2593 which is the average interval (in log units) between the von Frey monofilaments, and y is a value that depends upon the pattern of withdrawal responses. This procedure was repeated three times, and the average of the three trials was recorded as the animal's mean mechanical paw withdrawal threshold (MPWT).

The response to acute thermal pain was assessed using the Hargreaves method (Hargreaves et al., 1988). In this behavioral test, animals were placed in a Plexiglas chamber (20 x 15 x 40.5 cm) atop a raised clear Plexiglas floor to habituate for 20 minutes. Below the Plexiglas floor, there was a device that emittted an infrared beam of light when activated by the experimenter and shut off when the animal moved the paw located directly above the beam. Triggering the beam started a timer, which was also stopped when the animal removed its paw. Three trials were conducted for each paw, with at least 10 minutes between to prevent sensitivity to the thermal stimulus.

Pain Affect

The emotional response to noxious stimulation during the withdrawal period was assessed using the place escape/avoidance paradigm (PEAP, LaBuda & Fuchs, 2000a, 2000b, 2001, 2005). For this test, animals were given a .05 ml injection of 1% carrageenan in normal saline (or saline alone) in the plantar left hind paw with a 30 gauge needle. Carrageenan is an inflammatory agent that induces swelling, redness, and hypersensitivity in the area surrounding the injection (Winter et al., 1962). Three hours and forty-five minutes after the injection, MPWT and thermals were evaluated again and immediately followed by PEAP testing. The animal was placed in a 60 x 30 x 30 cm Plexiglas chamber with a light and dark side (painted white and black, respectively) located on an elevated platform with a mesh screen to access the hind paws during testing. The animal was stimulated with the highest von Frey monofilament on the plantar surface of its paws every fifteen seconds. If the subject was located on the dark side of the chamber, the left paw was stimulated, and if it was on the light side of the chamber, the right paw was stimulated. The animal's location in the chamber and the number of crosses was recorded for each fifteen second interval during the course of the thirty-minute testing period. This test was designed to examine pain affect by quantifying escape/avoidance behavior in response to noxious stimulation. The animals naturally prefer the dark half of the chamber, due to the perception of safety in the darkness. When presented with this novel environment, normal

animals will remain almost exclusively on the dark side of the chamber. On the other hand, animals with an experimentally induced pain condition will cross over to the light half more often (as a means of escaping the painful stimulus), eventually remaining on this side for the majority of the time to avoid the aversive stimulation. Testing for mean paw withdrawal thresholds establishes the level of sensory pain (i.e., the nociceptive stimulus clearly evokes sensory nociceptive responses) and the place escape/avoidance paradigm assesses how bothersome the pain of the stimulation is by observing whether the animal is willing to actively avoid the associated area. Drugs and manipulations that decrease the amount of pain affect (with or without lowering sensory pain) lead to a reduction in escape/avoidance behavior. Lesions of the anterior cingulate cortex (ACC), a brain region critical in processing of the affective component of pain, nearly abolish escape/avoidance behavior without altering sensory thresholds (LaGraize et al., 2004b). Morphine administration also decreases the amount of escape/avoidance behavior compared to saline controls (LaBuda & Fuchs, 2000a).

Procedure

Schedule of Drug Injections

The use of incremental dosing schedules in previous studies has produced consistent levels of tolerance and withdrawal following abstinence or precipitated by opioid antagonists (Kest et al., 2000; Abbott et al., 1982; Mucha et al., 1978). The doses selected for the current study were able to produce an adequate withdrawal response without risking the loss of animals to respiratory difficulty or severe weight loss (Ercoli & Lewis, 1945; Mucha et al., 1978 & 1979).

Subjects received subcutaneous injections of morphine or normal saline with a 27 gauge needle at a dose of 1 ml/kg at 10:00AM and 10:00PM on Days 1 through 6, with a final 10:00 AM injection on Day 7. The concentration of morphine was increased in the following pattern: Day 1: 10 mg/kg, Day 2: 10 mg/kg, Day 3: 15 mg/kg, Day 4: 15 mg/kg, Day 5: 15 mg/kg, Day 6: 20 mg/kg, Day 7: 20 mg/kg (dosed at 1 ml/kg). Withdrawal assessments occurred on Day 8, twenty-four hours after the final AM dose on Day 7. Spontaneous withdrawal was

preferable for this experimental protocol because the syndrome develops over several hours (12-24 hours abstinence) and lasts for days, while naloxone-precipitated withdrawal is brief (less than 24 hours) and the somatic symptoms are far more intense, which makes behavioral testing difficult (Frenois et al., 2002; Mucha, 1987).

Following weaning at 30 days of age, animals were randomly assigned to one of two housing groups: normal group housing (3-4 animals per cage) or social isolation (1 animal per cage). Previous work in our laboratory has demonstrated that early life stress in rats leads to enhanced pain affect in adulthood (Uhelski & Fuchs, 2010), and social isolation has been shown to induce an up-regulation in opioid receptors expression in selected brain regions, including the substantia nigra and ventral tegmental areas (Vanderschuren et al., 1995; DeVries et al., 2003; Miczek et al., 2008). Clinical studies suggest that early life trauma is associated with a number of mood disorders as well as an increased vulnerability to drug abuse and addiction (Finestone et al., 2000).

Upon reaching adulthood (60 days of age), subjects began the experimental protocol. On Day 0 (Baseline), Baseline MPWT and thermal latencies were assessed. Previous work in our laboratory showed that early life stress did not significantly alter sensory thresholds (Uhelski & Fuchs, 2010), and thus our social isolation procedure was not expected to alter the response to MPWT or thermal testing at Baseline. Starting on Day 1, animals received daily injections of morphine or normal saline at 10:00 am and 10:00 pm for six days, with the following incremental doses: Day 1: 10 mg/kg, Day 2: 10 mg/kg, Day 3: 15 mg/kg, Day 4: 15 mg/kg, Day 5: 15 mg/kg, Day 6: 20 mg/kg, Day 7: 20 mg/kg (dose remained at 1 ml/kg). On Day 7, the AM injection was followed by abstinence in order to induce spontaneous withdrawal. MPWT and thermal responses were evaluated one hour after the AM dose on Day 1 and Day 6 to ensure that an adequate analgesic response was present in animals receiving chronic morphine. On Day 8, a withdrawal assessment, MPWT and thermal testing were conducted 24 hours after the final dose. Upon completion of sensory testing, animals received an injection of either 1%

carrageenan or normal saline. After 3.5 hours, MPWT and thermals were evaluated again just prior to place escape/avoidance testing (PEAP). These sensory and affective tests were performed again on Day 9 (48 hours of abstinence) and Day 12 (120 hours abstinence).

Table 2.1 Outline of Experimental Procedures

	Day	Event
Dosing Period	Day 0 (Baseline)	MPWT Thermals
	Day 1	AM Dosing PM Dosing MPWT Thermals
	Day 2	AM Dosing PM Dosing
	Day 3	AM Dosing PM Dosing
	Day 4	AM Dosing PM Dosing
	Day 5	AM Dosing PM Dosing
	Day 6	AM Dosing PM Dosing MPWT Thermals
	Day 7	AM Dosing
Withdrawal Period	Day 8	Withdrawal Assessment MPWT Thermals Carrageenan Injection MPWT Thermals PEAP
	Day 9 & 12	MPWT Thermals PEAP

CHAPTER 3

RESULTS

3.1 Weight Data

Subjects were weighed twice daily on Days 1 through 6, then once on Days 7-12. For ease of analyses, AM and PM weights on Days 1 through 6 were averaged to produce a mean daily weight. A repeated measures analysis of variance (ANOVA) was used to analyze the impact of Drug, Housing, and Pain condition on body weight over the course of the experimental protocol. There was a significant main effect for drug condition, $F(1,69) = 4.53$, $p<.01$, and time, $F(11, 759) = 84.84$, $p<.001$, along with significant interactions for drug by time, $F(11, 759) = 182.18$, $p<.001$, pain by time, $F(7, 759) = 6.29$, $p<.001$, and drug by housing by time, $F(11, 759) = 2.38$, $p<.01$, and a marginally significant housing by time interaction, $F(11, 759) = 1.80$, $p=.05$. No other significant main effects or interactions were detected. Post-hoc analyses (Fisher's LSD) revealed that morphine-dosed subjects had significantly lower body weights starting on Day 8 and continuing through Day 12, despite a recovery in the latter days of the withdrawal period. Saline-dosed subjects showed significant increases in body weight on Days 2 through 12, regardless of housing condition, but changes in body weight differed between group- and single-housed morphine subjects. Single-housed, morphine-dosed subjects failed to gain weight during the dosing period and had significantly lower body weights relative to single-housed, saline-dosed subjects on Days 5, 6, and 8 through 11. Group-housed, morphine-dosed subjects also failed to gain weight relative to Day 1, and mean body weights differed significantly from single-housed, saline-dosed subjects on Days 7 through 12.

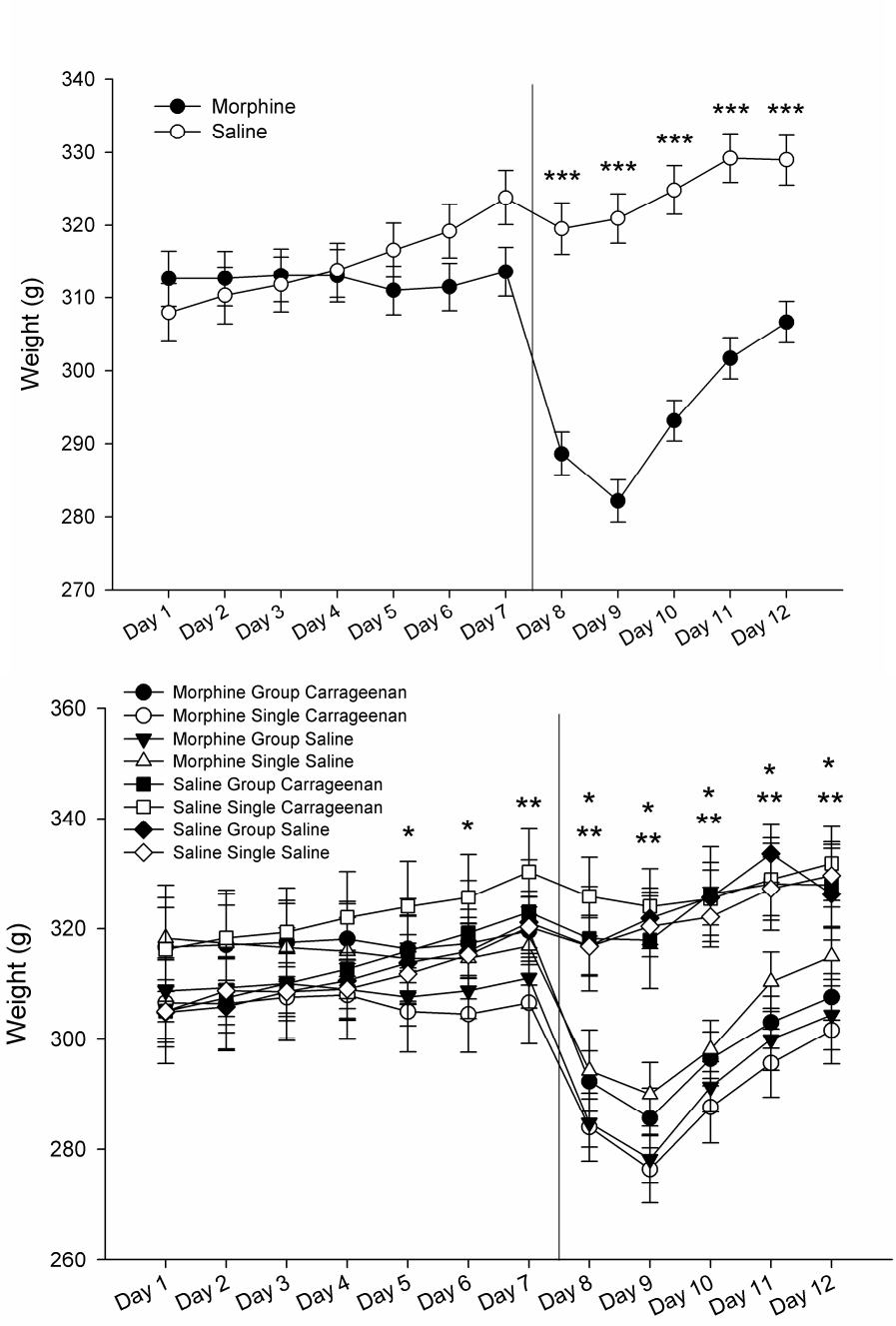

Figure 3.1 Daily weights of subjects in grams. (a) Weights for each Drug condition over the twelve-day experimental protocol, and (b) Weights for Drug and Housing Condition over the twelve-day experimental protocol. *** p<.001 (relative to saline-dosed subjects), **p<.005 (for group-housed morphine subjects relative to group-housed saline subjects) *p<.05 (for single-housed morphine subjects relative to singe-housed saline subjects)

3.2 Withdrawal Assessments

The severity of the withdrawal syndrome was assessed 24 hours following the final dose of morphine, immediately before testing for sensory thresholds. Each subject was assigned a withdrawal score based on the presence or absence of four physical withdrawal symptoms: teeth chattering, piloerection, diarrhea, and ptosis. For data analysis, symptoms were analyzed individually and using the overall withdrawal score representing the number of positive symptoms. Only one subject received a score of 4, and so four categories were created for analysis: 0, 1, 2 and 3+. Individual Chi-Square analyses were performed to examine the impact of Drug and Housing condition. The effects of drug and housing alone were assessed where significant overall differences were present among the Drug + Housing conditions.

Withdrawal scores differed significantly among the four conditions (group housed + morphine, group housed + saline, single housed + morphine, single housed + saline), $X^2(9) = 51.60$, $p<001$. Further analyses revealed that while drug condition had a significant effect, $X^2(3) = 39.20$, $p<.001$, housing condition alone did not significantly impact withdrawal scores, $X^2(3) = 5.08$, n.s. Overall, subjects dosed with morphine demonstrated significantly higher withdrawal scores than saline-dosed controls, regardless of housing condition.

Table 3.1 Distribution of Withdrawal Scores

	Observed Frequency			
Condition	0	1	2	3+
Group Morphine	3	7	9	1
Group Saline	14	4	1	0
Single Morphine	2	1	9	6
Single Saline	15	5	0	0
Total	**34**	**17**	**19**	**7**

An overall effect of Drug + Housing condition was present for three of the four withdrawal symptoms. The presence or absence of teeth chattering was dependent upon the subject's condition, $X^2(3) = 31.72$, $p<.001$. Further analyses demonstrated that this effect was a

results of the subjects' Drug condition, $X^2(1) = 29.58$, p<.001, but not impacted by housing condition, $X^2(1) < 1$, n.s. Significantly more morphine-dosed subjects demonstrated teeth chattering relative to saline-dosed subjects. This same pattern emerged for ptosis, where overall condition had a significant impact, $X^2(3) = 21.36$, p<.001, with Drug condition driving the effect, $X^2(1) = 17.86$, p<.001, but not housing, $X^2(1) < 1$, n.s. Piloerection was dependent on overall condition, $X^2(3) = 57.79$, p<.001, with a significant effect for both drug, $X^2(1) = 17.86$, p<.001, and housing condition, $X^2(1) = 22.52$, p<.001. Morphine-dosed subjects were rated positively for piloerection significantly more than saline-dosed subjects, and single-housed subjects were more likely to demonstrate piloerection relative to group-housed subjects. The presence or absence of diarrhea was not found to be dependent on overall condition; $X^2(1) = 3.38$, n.s, thus, no further analyses were performed.

Table 3.2 Distribution of Individual Withdrawal Symptoms

	Symptom							
	Teeth Chattering		Piloerection		Diarrhea		Ptosis	
Condition	Yes	No	Yes	No	Yes	No	Yes	No
Group Morphine	12	8	1	19	3	17	12	8
Group Saline	2	17	0	19	3	16	1	18
Single Morphine	15	3	17	1	2	16	6	12
Single Saline	2	18	2	18	0	20	1	19
Total	**31**	**46**	**20**	**57**	**8**	**69**	**20**	**57**

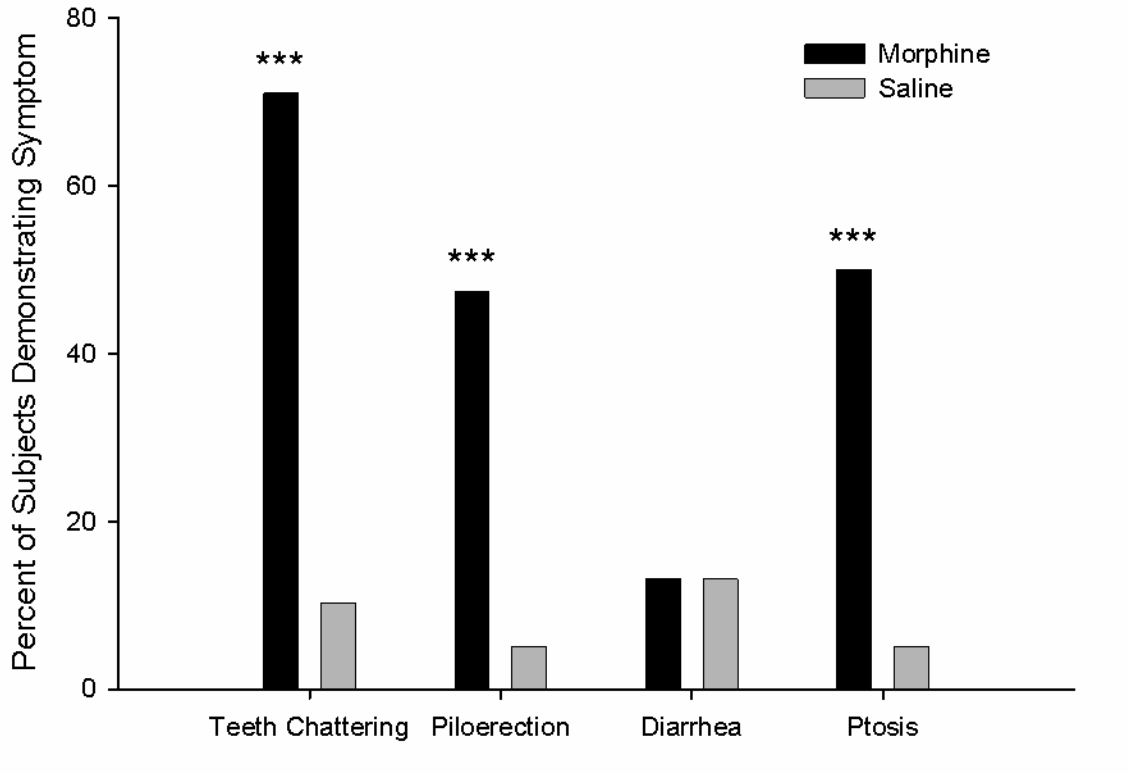

Figure 3.2 Percent of subjects demonstrating withdrawal symptoms for each Drug condition.***p<.001

Three behavior categories were also recorded during the ten-minute withdrawal assessment: rearings, grooming, and wet dog shakes. Analyses of variance (ANOVA) were performed to examine the impact of Drug and Housing condition on each behavior category separately. For rearing behaviors, analysis revealed a significant main effect for Drug, $F(1, 73) = 40.93$, $p<.001$, and a significant Drug by Housing interaction, $F(1, 73) = 6.14$, $p<.05$. Post-hoc analyses revealed that morphine-dosed rats demonstrated significantly lower rearing behaviors compared to saline-dosed rats, regardless of housing condition. For grooming and wet dog shakes, there were no significant main effects or interactions present.

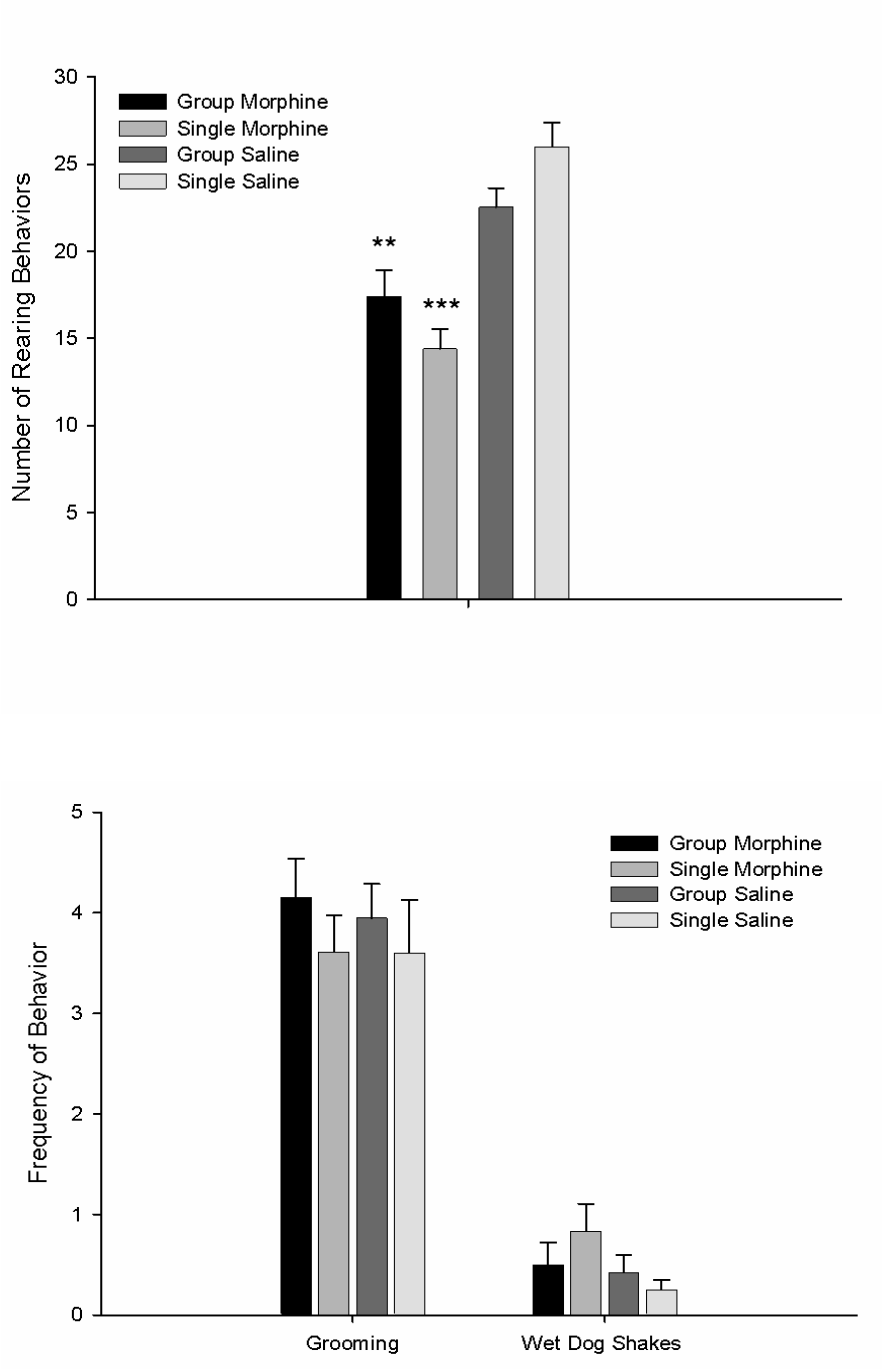

Figure 3.3 Behaviors observed during withdrawal assessment. (a) Rearing behaviors and (b) Grooming and wet dog shakes. ***p<.001, **p<.01 (relative to saline-dosed rats)

3.3 Assessments of Sensory Thresholds

Behavioral assessments of sensory thresholds and the response to noxious thermal stimulation were assessed at Baseline, one hour after AM dosing on Days 1 and 6, on Day 8 following withdrawal assessments and 3.5 hours after carrageenan or saline injections, and on Days 9 and 12.

3.3.1 Thermal Withdrawal Latencies

The mean withdrawal latency of both hind paws was used to assess the impact of Drug and Housing condition on the response to noxious thermal stimulation at Baseline, Day 1, Day 6, and Day 8-1 pre-carrageenan. Repeated measures ANOVA revealed significant main effects for Drug, $F(1,73) = 102.73$, $p<.01$, Housing, $F(1,73) = 6.07$, $p<.05$, and time, $F(3, 219) = 57.99$, $p<.001$, with a significant Drug by time interaction, $F(3, 219) = 50.39$, $p<.001$. Post-hoc analyses (Fisher's LSD) showed that all subjects demonstrated similar Baseline latencies, but those dosed with morphine had significantly higher latencies relative to saline-dosed subjects on Day 1 and Day 6, but not Day 8 pre-carrageenan. Morphine demonstrated significant analgesic efficacy acutely, but this effect was tempered after chronic dosing, suggesting that repeated dosing led to the development of opioid tolerance by Day 6. There was no evidence that opioid withdrawal altered thermal latencies on Day 8, as these values were not significantly different from baseline. Housing condition had a slight impact on thermal latencies, with group-housed subjects overall demonstrating significantly higher latencies than single-housed subjects on Day 1 and Day 8 pre-carrageenan.

The four assessments during the withdrawal period (Day 8 pre- and post-carrageenan, Day 9, and Day 12) were analyzed using data from the left hind paw only. Right hind paw latencies remained at or near Baseline levels, and were not analyzed further (data not shown). To evaluate the impact of withdrawal and the efficacy of carrageenan injections, left paw

latencies were converted to percent change scores representing the magnitude of change from Baseline latencies, with positive values reflecting decreased latencies and negative values reflecting increased latencies. Repeated measures ANOVA with Drug, Housing, and Pain condition revealed significant main effects for Pain, $F(1,69) = 28.09$, $p<.001$, and time, $F(3, 207) = 33.10$, $p<.001$, as well as a Pain by time interaction, $F(3, 207) = 35.81$, $p<.001$. No other significant main effects or interactions were detected. Overall, subjects injected with saline demonstrated significantly lower percent change scores relative to carrageenan-injected subjects on Day 8 post-injection, Day 9 and Day 12. This pattern confirms that the carrageenan injections induced hypersensitivity that remained throughout the withdrawal period; however, the magnitude of the effect decreased slightly over time. No impact from morphine withdrawal or housing condition was present in thermal withdrawal latencies.

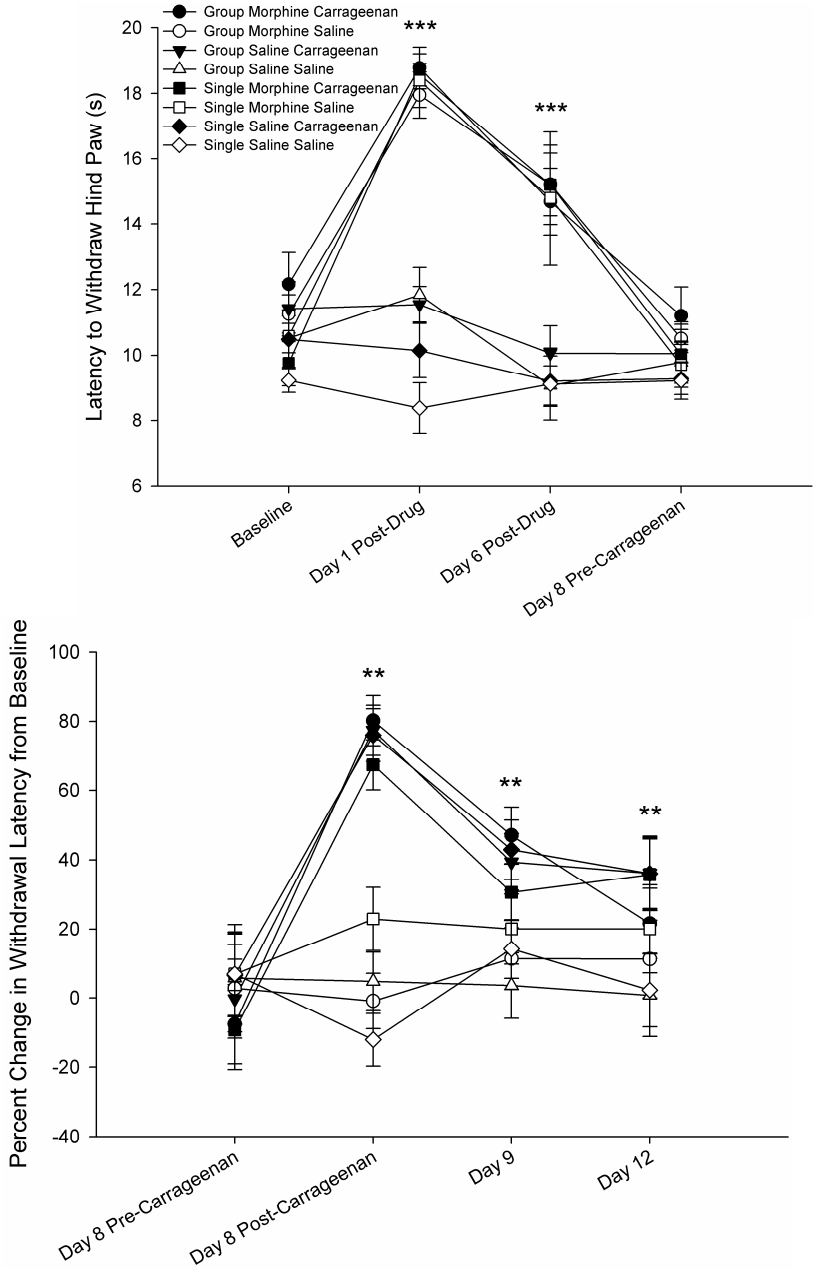

Figure 3.4 Changes in thermal withdrawal latencies over time for (a) both paws prior to carrageenan injections and (b) percent change from Baseline for the left paw only during the withdrawal period. ***p<.001 (for morphine-dosed rats relative to Baseline latencies), **p<.005 (for carrageenan-injected rats relative to saline-injected rats)

3.3.2 Mechanical Paw Withdrawal Thresholds

The mean threshold of both hind paws was used to assess the impact of Drug and Housing condition on threshold values at Baseline, Day 1, Day 6, and Day 8-1 pre-carrageenan. Almost all subjects demonstrated maximum mean paw withdrawal thresholds (MPWT) at Baseline, and remained high for all assessments prior to carrageenan injections on Day 8. This ceiling effect precludes parametric analyses due to the lack of variance among subjects. Chi-Square analyses were performed with Drug and Housing condition using two dependent categories (maximum threshold or less than maximum threshold). The analyses revealed a significant effect for Drug condition on Day 8 pre-carrageenan only, $X^2(1) = 7.78$, p=.005, and a significant effect for Housing condition at Baseline, X^2 (1) = 7.90, p=.005. Single-housed subjects had significantly more non-maximal thresholds at Baseline relative to group-housed subjects, and morphine-dosed subjects had significantly more non-maximal threshold values than saline-dosed subjects on Day 8 pre-carrageenan. However, overall mean threshold values were less than 10% from maximal values, indicating that the magnitude of the group differences did not provide evidence of mechanical hypersensitivity prior to carrageenan injections.

For assessments during the withdrawal period (Days 8, 9, and 12), threshold values for the right hind paw remained near Baseline levels (data not shown), and thus were not analyzed further. Thus, only changes in the injected left hind paw were examined to assess the impact of Drug, Housing, and Pain condition on threshold values over time (four time points: Day 8 pre- and post-carrageenan, Day 9 and Day 12). Threshold values were converted to percent changes scores representing the magnitude of change in threshold value from Baseline thresholds, with positive values reflecting decreased thresholds and negative values reflecting increases in thresholds. Repeated measures ANOVA was performed to examine the effect of drug, housing, and pain condition on threshold changes over time (Four time points: Day 8 pre- and post-carrageenan, Day 9, and Day 12). The analysis revealed significant main effects for

Pain condition, $F(1, 69) = 356.49$, $p<.001$, and time, $F(3, 207) = 140.68$, $p<.001$, as well as significant interactions for Pain condition by time, $F(3, 207) = 151.90$, $p<.001$, and Drug condition by Pain condition by time, $F(3, 207) = 3.44$, $p<.05$. No other main effects or interactions were found to be significant. Post-hoc analyses revealed that carrageenan-injected subjects demonstrated significantly higher change scores relative to saline-injected subjects on Day 8 (post-carrageenan), 9 and 12, which confirms the presence of mechanical hypersensitivity following the injection of the inflammatory agent, carrageenan which persisted throughout the withdrawal period. However, the magnitude of the effect decreased over time, with significant decreases in change scores on Day 9 and Day 12.

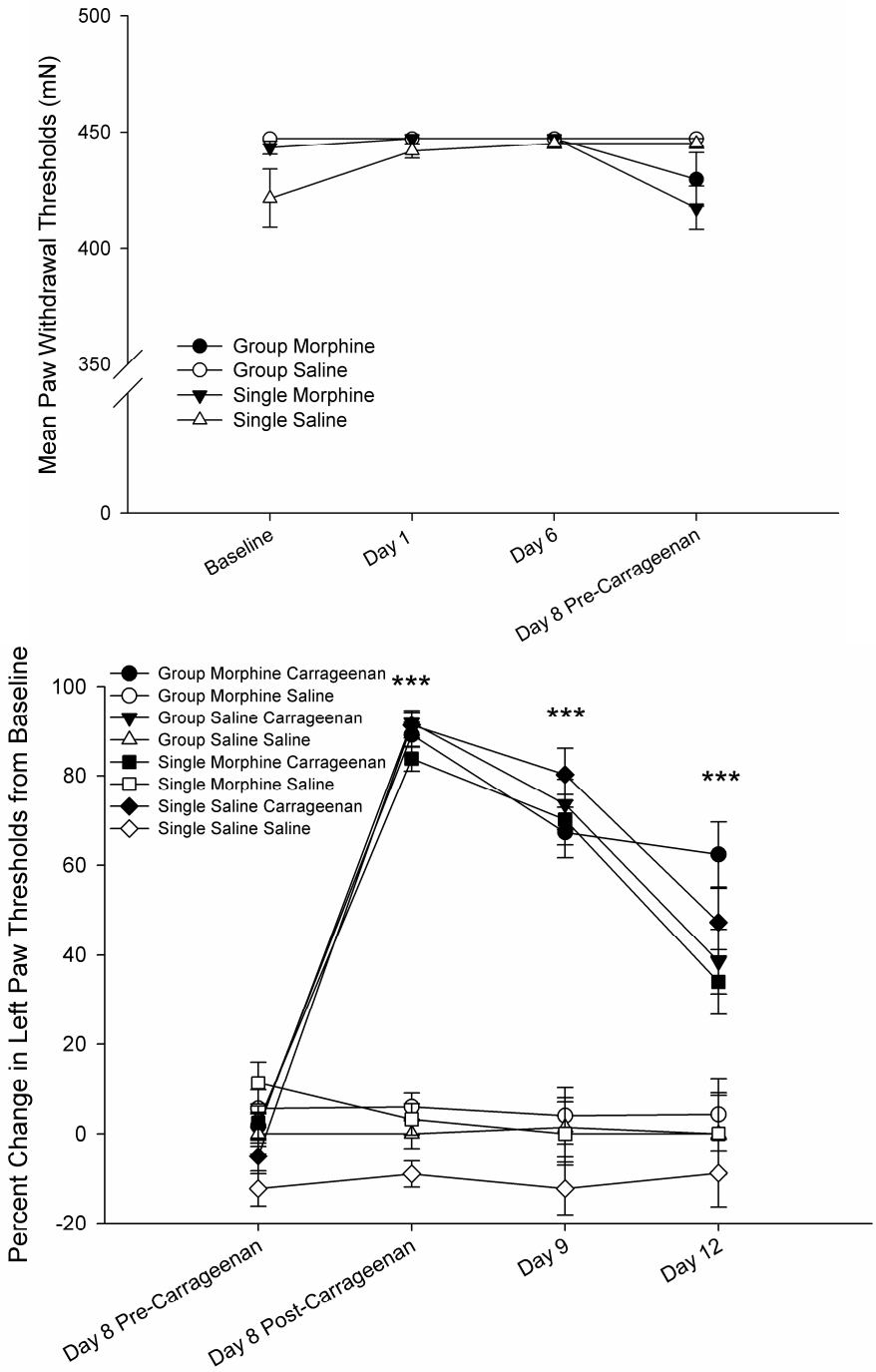

Figure 3.5 Mean paw withdrawal thresholds assessed (a) for both paws prior to carrageenan injections and (b) for the left paw only during the withdrawal period using percent change from Baseline values. ***p<.001 (for carrageenan-injected rats relative to saline-injected rats)

3.4 Assessments of Pain Affect

Subjects were tested using the Place Escape/Avoidance Paradigm (PEAP) during the withdrawal period following post-carrageenan sensory assessments on Day 8 and again following sensory assessments on Days 9 and 12. The percent of time spent in the light side of the PEAP chamber as well as the number of midline crosses were calculated for each five-minute period during the thirty-minute testing period, forming six 5-minute time bins (5, 10, 15, 20, 25, and 30 min) for analyses of the impact of Drug, Housing, and Pain condition for each testing day. An overall average for the percent of time spent in the light side of the chamber was also calculated for analysis of changes in escape/avoidance behavior over the three testing days.

For Day 8, repeated measures ANOVA revealed a significant main effect for Pain, $F(1,69) = 30.40$, $p<.001$, and significant interactions for Drug by time, $F(5, 345) = 3.31$, $p<.01$, Pain by Housing, $F(1, 69) = 4.15$, $p<.05$, and Pain by time, $F(5, 345) = 9.98$, $p<.001$. No other significant main effects or interactions were present. Post-hoc analyses (Fisher's LSD) were performed to examine significant interactions. Among morphine-dosed subjects, group-housed subjects injected with carrageenan spent significantly more time in the light side of the chamber relative to those injected with saline for all time points. For single-housed rats dosed with morphine, carrageenan-injected rats spent significantly more time in the light only during the latter half of the testing period (20 , 25 and 30 min). Saline-dosed subjects demonstrated a slightly different pattern of avoidance. Group-housed rats injected with carrageenan spent more time in the light relative to rats injected with saline during the first time point and the latter half of the test (5, 20, 25 and 30 min), while single-housed rats injected with carrageenan only spent more time in the light than those injected with saline during the final third of the test (25 and 30 min). Finally, morphine-dosed subjects injected with carrageenan spent significantly more time

in the light during the latter half of the testing period (20 and 25 min) relative to those injected with saline.

For Day 9, repeated measures ANOVA revealed a significant main effect for Pain, $F(1,69) = 62.96$, $p<.001$, and significant interactions for Pain by Housing, $F(1, 69) = 5.85$, $p<.05$, and Pain by time, $F(5, 345) = 17.28$, $p<.001$. No other significant main effects or interactions were present. Post-hoc analyses (Fisher's LSD) were performed to examine significant interactions. Among carrageenan-injected subjects, group housed subjects spent significantly more time in the light side of the chamber relative to single housed subjects, regardless of Drug condition. No differences in escape/avoidance behavior were detected among the saline-injected subjects.

For Day 12, repeated measures ANOVA revealed significant main effects for Pain, $F(1,69) = 19.83$, $p<.001$, and time, $F(5, 345) = 4.79$, $p<.001$, as well as a significant interaction for Pain by time, $F(5, 345) = 8.80$, $p<.001$. No other significant main effects or interactions were present. Post-hoc analyses (Fisher's LSD) were performed to examine significant interactions. Overall, carrageenan-injected subjects spent more time in the light side of the chamber relative to saline-injected subjects, regardless of Drug or Housing condition. Saline-injected subjects did show a significant decrease in time spent on the light side over the course of the testing period, but carrageenan-injected subjects did not.

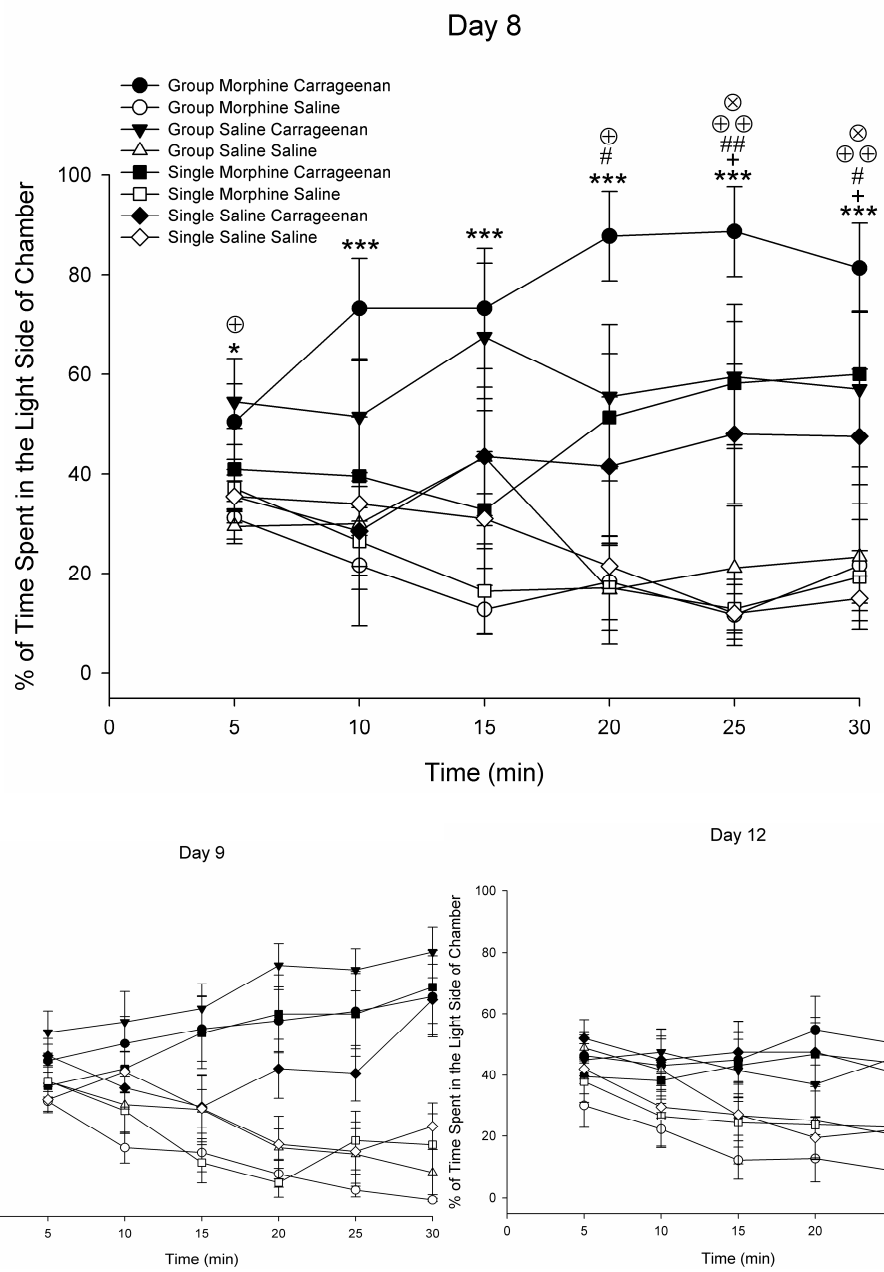

Figure 3.6 Percent of time spent in the light side of the chamber over the thirty-minute testing period on (a) Day 8, (b) Day 9, and (c) Day 12. *p≤.05,
**p<.01, ***p≤.001 (for group housed morphine-dosed carrageenan rats relative to saline rats)
+p≤.05 (for group housed morphine-dosed carrageenan rats relative to group housed saline-dosed carrageenan rats)
#p<.05, ##p<.01 (for single housed morphine-dosed carrageenan rats relative to saline rats)
$\oplus$ p≤.05, $\oplus\oplus$ p<.01 (for group housed saline-dosed carrageenan rats relative to saline rats)
$\otimes$ p<.01 (for single housed saline-dosed carrageenan rats relative to saline)

The impact of Drug, Housing, and Pain condition on escape/avoidance behavior over the three testing days was also assessed using the mean percent of time spent in the light side of the chamber for each subject on Day 8, 9, and 12. Repeated measures ANOVA revealed a significant main effect for Pain, $F(1, 69) = 68.41$, $p<.001$, and significant interactions for Pain by Housing, $F(1, 69) = 5.52$, $p<.05$, and Drug by Housing by Pain by time, $F(2, 138) = 3.07$, $p = .05$. Post-hoc analyses (Fisher's LSD) showed that carrageenan-injected subjects spent more time in the light side of the chamber than saline-injected subjects at all three time points for group housed subjects, regardless of Drug condition. A different pattern emerged for single housed subjects, with morphine-dosed carrageenan subjects spending more time in the light on Day 8 and 9 only and saline-dosed carrageenan subjects spending more time in the light on Day 9 and 12 only.

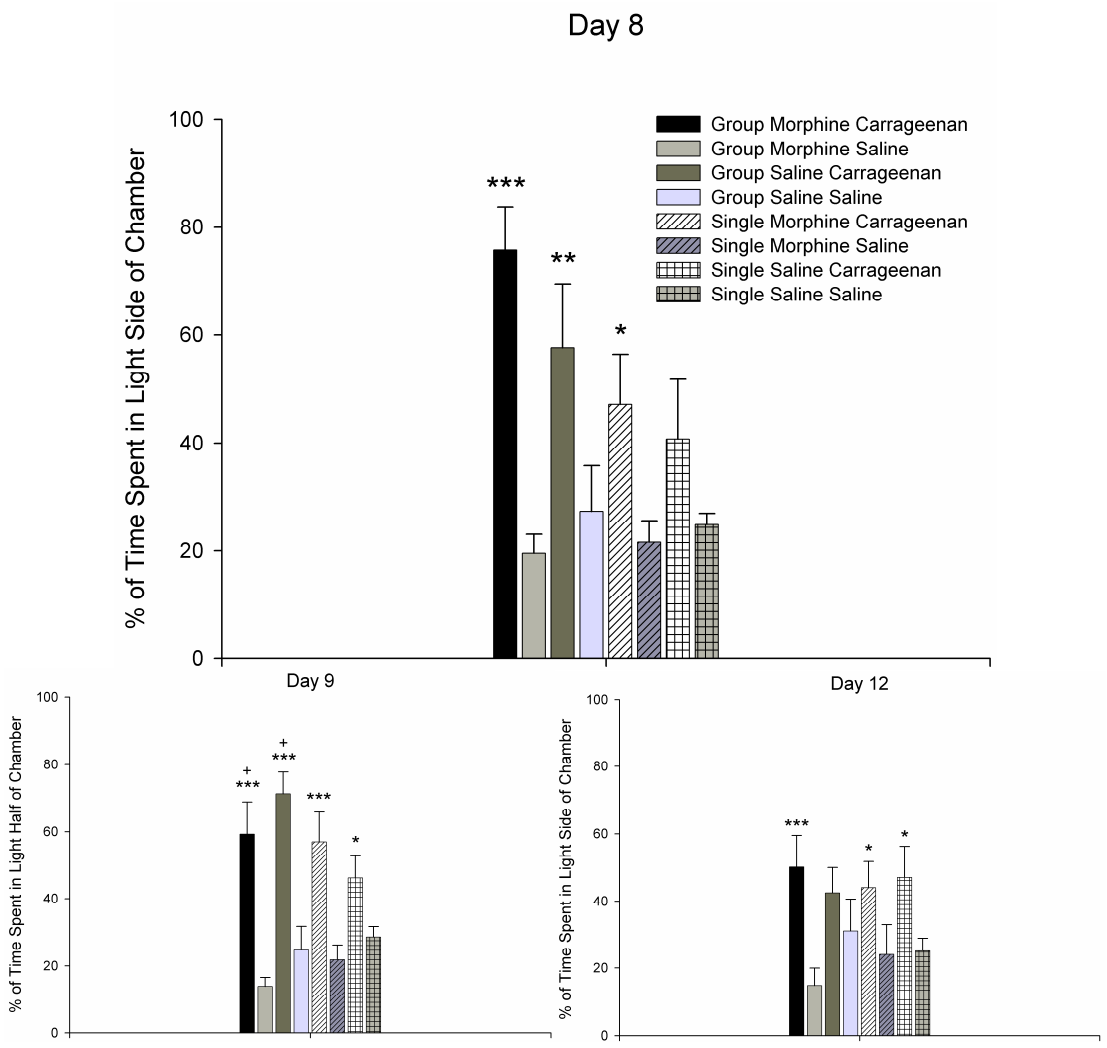

Figure 3.7 Mean percent of time in the light half of the chamber for (a) Day 8, (b) Day 9, and (c) Day 12. ***p<.001, **p<.01, *p≤.05 (relative to saline-injected rats), $^{+}$p<.05 (relative to Day 8)

The number of midline crosses was assessed using the mean number of crosses on Day 8, 9 and 12 to assess movement in the chamber as influenced by Drug, Housing, and Pain condition over time. Repeated measures ANOVA revealed significant main effects for Drug, $F(1, 69) = 23.82$, $p<.001$, Housing, $F(1, 69) = 21.81$, $p<.001$, Pain, $F(1, 69) = 7.21$, $p<.01$, time, $F(2, 138) = 4.53$, $p<.05$, and significant interactions for Drug by Housing, $F(1, 69) = 8.33$, $p<.01$, and Pain by time, $F(2, 138) = 25.96$, $p<.001$. Post-hoc analyses revealed that carrageenan-injected subjects demonstrated significantly more midline crosses on Day 9 and 12 relative to Day 8, while saline-injected subjects performed significantly fewer midline crosses on Day 9 and 12 relative to Day 8. In addition, carrageenan-injected subjects performed significantly fewer midline crosses relative to saline-injected subjects on Day 8. Overall, single-housed subjects dosed with morphine crossed the midline significantly less often than saline-dosed subjects, but no differences in crossing behavior were present between Drug conditions for group-housed subjects.

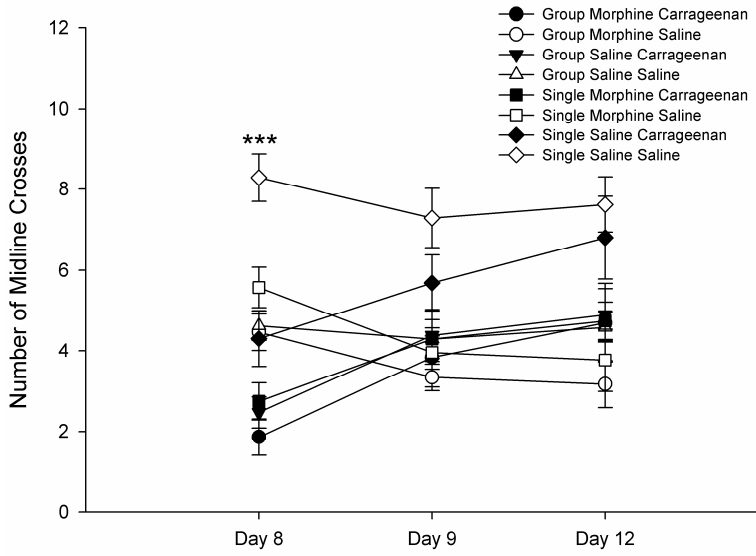

Figure 3.8 Mean number of midline crosses over the three day PEAP testing period. ***p<.001 (for carrageenan-injected subjects relative to saline-injected subjects)

CHAPTER 4

DISCUSSION

4.1 Weight Data

The use of daily body weights provided a straightforward measure for the impact of morphine dosing and the withdrawal period on overall physical functioning. Regardless of Housing or Pain Condition, saline-dosed subjects gained weight significantly throughout the experimental protocol, indicating that these manipulations did not have a negative impact on weight gain in these subjects. Conversely, morphine-dosed subjects failed to gain weight during the dosing period, and began to demonstrate significant weight loss by the end of dosing. Housing condition influenced the pattern of weight loss during Days 5-7, with single-housed subjects beginning a decline on Day 5 and group-housed subjects on Day 7. Regardless of Housing condition, all morphine-dosed subjects experienced significant weight loss during the withdrawal period. The decline began to reverse on Day 10, but had not recovered completely by Day 12. This pattern corresponds to the timing of the spontaneous withdrawal syndrome, where physical symptoms first appear 12 to 24 hours after the last dose and persist for around 72 hours (Xiao et al., 2009). Body weight data from this study demonstrated a significant impact of Drug condition on morphine subjects during the dosing and withdrawal period, confirming that the length of dosing and concentrations selected were able to produce a reliable spontaneous withdrawal syndrome.

4.2 Withdrawal Assessments

The severity of the physical withdrawal syndrome and its impact on behavior were evaluated 24 hours after the final dose of morphine. Although not as severe as precipitated withdrawal, morphine-dosed subjects in the current study demonstrated a clear spontaneous withdrawal response. Morphine-dosed subjects displayed teeth chattering, piloerection and ptosis more often than saline-dosed subjects, but few subjects were found to have diarrhea. Interestingly, Housing condition had a significant impact on whether morphine-dosed subjects would show piloerection during the withdrawal assessment, with far more single-housed subjects demonstrating this symptom relative to group-housed subjects. Previous studies have shown that social isolation during early life enhances stress reactivity during adulthood, and so this symptom may have reflected a difference in stress levels between group and single housed subjects (Tsoory et al., 2007; Lukkes et al., 2009).

No differences were detected for grooming behavior or wet dog shakes during the withdrawal assessment, but these symptoms are reported more often during precipitated withdrawal and may not be altered by the less intense spontaneous withdrawal syndrome. There was a significant Drug effect on rearing behaviors, with saline-dosed subjects performing significantly more rearing behaviors during the assessment when compared to morphine-dosed subjects.

Overall, a reliable physical withdrawal syndrome was induced by abstinence following the dosing regimen in this study. Not all aspects of physical and behavioral assessments were altered, which suggests that morphine-dosed subjects were experiencing a withdrawal syndrome that was not as severe as those seen using precipitated withdrawal. The milder withdrawal syndrome was desirable in the current study, since subjects were not so physically ill that behavioral assessments would be confounded (Frenois et al., 2002; Mucha, 1987).

4.3 Assessments of Sensory Thresholds

Assessments of response to noxious thermal stimulation at Baseline did not shown any overall differences among the conditions, as expected. The post-drug assessment on Day 1 showed a robust analgesic effect for acute morphine, regardless of Housing group. The analgesic effect was also present following chronic morphine, as seen post-drug on Day 6, but the effect was clearly mitigated by tolerance from repeated dosing. Although incremental dosing was utilized in an attempt to prevent the development of tolerance, many subjects did develop tolerance by Day 6. Fortunately, there was still a strong analgesic effect that was not entirely reversed by tolerance.

No differences among the conditions were detected on Day 8 prior to carrageenan injections, which contradicts our prediction that withdrawal would induce lower thermal latencies in morphine-dosed subjects. Mean response latencies returned to Baseline levels regardless of Drug or Housing condition. Following carrageenan injections, there was a significant effect of Pain condition on Days 8, 9 and 12, but no detectable differences among Drug or Housing conditions. Contrary to our hypothesis, morphine withdrawal did not alter thermal hypersensitivity in response to carrageenan inflammation.

Prior to carrageenan injections, no robust effects for Drug condition were detected. This is not entirely unexpected, as ceiling effects at Baseline do not allow for increases in threshold following the administration of analgesics. Contrary to our hypothesis, no hypersensitivity was detected following morphine abstinence on Day 8, suggesting that opioid withdrawal does not significantly alter the response to mechanical stimuli in subjects without an experimental pain condition. Housing condition also failed to alter mechanical paw withdrawal thresholds prior to carrageenan injections, as predicted. Following carrageenan injections, all subjects injected with the inflammatory agent demonstrated significantly lower mechanical paw withdrawal thresholds, decreasing 50% or more from Baseline values. This confirms that the experimental pain manipulation produced a reliable inflammatory state which resulted in mechanical

hypersensitivity starting 3.5 hours after the injection and persisting until the final assessments on Day 12. While there were individual differences among the Drug and Housing conditions over time, there was not a robust impact of either condition on mean paw withdrawal thresholds post-carrageenan, contrary to what was predicted. Assessments of mechanical hypersensitivity were unable to detect an effect for morphine withdrawal regardless of Pain condition.

4.4 Assessments of Pain Affect

Behavioral assessments of pain affect in response to carrageenan injections were performed during the withdrawal period on Days 8, 9 and 12. This was designed to allow two assessments during the early phase of the withdrawal period, when symptoms tend to be at their highest levels, and a third assessment at the very end of the withdrawal phase, when symptoms have abated (Xiao et al., 2009). Overall, there was a significant effect for Pain condition, with carrageenan-injected subjects spending significantly more time in the light side of the chamber compared to saline-injected subjects. This pattern replicates previous findings from our lab (LaBuda & Fuchs, 2000) and unpublished data examining the response to daily PEAP testing over a five-day period. In addition, Drug and/or Housing condition did alter the pattern of escape/avoidance over the course of the thirty-minute test on Day 8 and Day 9, but not Day 12, partially supporting our hypothesis. On Day 8, group-housed morphine-dosed carrageenan rats spent more time in the light side of the chamber compared to saline-dosed carrageenan rats during the latter half of the testing period. No differences in escape avoidance behavior were present among single-housed carrageenan subjects between the two Drug conditions. In addition, group-housed morphine-dosed carrageenan subjects spent significantly more time in the light side than single-housed, morphine-dosed carrageenan rats during the majority of the testing period (10-25 min). Taken together, these results suggest that the impact of morphine withdrawal on escape/avoidance behavior is significantly impacted by Housing condition, although in the opposite pattern from that which was predicted. Among subjects

injected with carrageenan, group-housed subjects demonstrated higher levels of pain affect than single-housed subjects, but only group-housed morphine-dosed subjects showed higher levels of pain affect relative to saline-dosed subjects. Housing condition also had an impact on Day 9, where carrageenan-injected group-housed subjects spent more time in the light side of the chamber relative to carrageenan-injected single-housed subjects, regardless of Drug condition. This pattern was not present on Day 12, where no differences were found among Drug and Housing conditions. This pattern of escape/avoidance behavior suggests that housing condition modified the response to the stress of noxious stimulation during early but not late withdrawal, with single-housed subjects preferring to remain in the dark relative to group-housed subjects. Despite the aversive nature of left paw stimulation, these subjects may have regarded the light side of the chamber as more aversive due to increased exposure in the presence of an injury. Highly anxious rodents are known to avoid exposed and lighted areas in open field and elevated plus mazes, and early life stress, including social isolation, has been demonstrated to produce such an effect (Holmes et al., 2005; Tsoory et al., 2007; Lukkes et al., 2009). The addition of morphine withdrawal may have compounded this effect, precluding any differences between single-housed subjects dosed with morphine versus saline. Thus, we were only able to detect enhanced pain affect in group-housed morphine subjects on Day 8. This effect did not persist on Day 9, which may reflect changes induced by reduction in withdrawal intensity or the fact that the PEAP environment was no longer novel. By Day 12, it was clear that group-housed, morphine-dosed subjects were no longer experiencing enhanced pain affect as the withdrawal syndrome waned.

Analyses of the number of midline crosses revealed that saline-injected subjects only crossed the midline significantly more times during the PEAPs testing period on Day 8, which likely reflected increased exploration for saline-injected subjects throughout the testing period and less exploration for carrageenan-injected subjects, which tended to investigate the chamber early on and remain on one side for the remainder of the test. Even though carrageenan-

injected subjects crossed the midline less often, our results were not a reflection of a complete absence of motor activity induced by the experimental pain condition. On Day 9, the intensity of the carrageenan inflammation and novelty of the PEAPs chamber had decreased, and no differences in midline crosses were present on this testing day or on Day 12.

4.5 General Discussion

The purpose of this study was to examine changes in both sensory and affective pain processing during the withdrawal period. We also evaluated the impact of social isolation stress on these behaviors. This study provides a new perspective in animal research models of addiction by explicitly evaluating both the sensory aspect of pain processing as well as the emotional response to noxious stimulation, the latter of which has not been previously examined.

The concentrations of morphine and length of the dosing regimen were adequate to produce robust analgesic effects during administration and a reliable spontaneous physical withdrawal syndrome following abstinence. Although our hypotheses regarding withdrawal-induced changes in sensory processing were not confirmed, these results were not entirely unexpected in light of previous studies examining similar reflexive responses during withdrawal. (Zissen et al., 2007; Sweitzer et al., 2004; Pinelli & Trivulzio, 1997). Although clinical studies in opioid addicts have suggested that sensory thresholds are decreased during withdrawal, behavioral assessments in rodents are hindered by their reflexive nature—while a patient can provide a clear verbal response, rodents are assessed using tail flick or paw withdrawal responses that may not involve supraspinal processing. Further studies examining sensory thresholds or nociceptive processing should endeavor to use assessments that do not rely entirely on reflexive responses, such as operant tasks that have been used to effectively evaluate nociceptive processing (Mauderli et al., 2000; LaGraize et al., 2004a; King et al., 2007).

The results revealed a clear alteration in escape/avoidance behavior over the three day testing period among Drug and Housing conditions. While group-housed, morphine-dosed carrageenan subjects spent more time in the light on average than saline-dosed carrageenan subjects on Day 8, this pattern reversed on Day 9, and by Day 12 no difference was present between the conditions. Interestingly, single-housed subjects performed in the opposite manner than expected, spending less time in the light side than group-housed subjects on Day 8 and 9. Although evidence of increased pain affect predicted for morphine-dosed subjects was seen on Day 8, social isolation did not enhance this effect—if anything, single-housed morphine subjects were more inhibited. This may represent an issue with the testing chamber itself—for normal animals, the dark if preferable unless it is associated with noxious stimulation, but for single-housed subjects, the dark remained preferable despite the unpleasantness. Future studies should attempt to assess pain affect during opioid withdrawal where light/dark or open/closed preferences are not utilized to prevent any impacts of enhanced anxiety-like behavior in socially isolated subjects.

Overall, the results of this study represent the first comprehensive assessment of pain processing during opioid withdrawal. While no changes in sensory pain processing were detected, there was clearly an effect of drug and housing condition on the emotional component of pain that provided evidence of enhanced pain affect in group-housed subjects in morphine withdrawal despite a lack of effect for single-housed subjects. The results of the current study could contribute to evaluations the efficacy of novel treatments for opioid addicts, especially those designed to reduce the intensity of withdrawal for chronic pain patients. If opioid withdrawal enhances the impact of an existing pain condition, the development of a reliable treatment beyond methadone and other agonists could assist with relapse prevention while also enabling the patient to abstain from opioids and recover with less distress.

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BIOGRAPHICAL INFORMATION

Megan L. Uhelski was born in Lansing, MI on April 26, 1984. She completed her undergraduate education at Baylor University in Waco, TX, earning a Bachelor of Science degree in Psychology in 2006, and earned her Master's Degree in Health Psychology from the University of Texas at Arlington in 2009. Her research focuses on behavioral analysis of various acute and chronic pain states and includes work with pharmacology and developmental aspects of pain processing. She is interested in studying the relationship between stress and pain mechanisms, as well as novel pharmacotherapy for chronic pain disorders.

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