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The Role of Pain and Anxiety in the Transition to Opioid Addiction

A dissertation submitted in partial satisfaction of the requirements for the degree  
Doctor of Philosophy

in

Biomedical Sciences

by

Paula Ehn Park

Committee in charge:

Professor George F. Koob, Chair  
Professor Vivian Hook, Co-Chair  
Professor William Joiner  
Professor Neal Swerdlow  
Professor Tony Yaksh

2013

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University of California, San Diego

2013

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## PUBLICATIONS

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M. Weber, R. Luedtke, WL. Chang, P.E. Park, J.P Durbin, R.H. Mach, N.R. Swerdlow, "Developing a prepulse inhibition-based screening assay for the detection of antipsychotic-like compounds with a D3 receptor linked mechanism of action." American College of Neuropsychopharmacology annual meeting, Scottsdale AZ, 12/2008

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WC10 and WC44. Pharmacology, Biochemistry and Behavior, 08/2009

ABSTRACT OF THE DISSERTATION

The Role of Pain and Anxiety in the Transition to Opioid Addiction

by

Paula Ehn Park

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor George F. Koob, Chair

Professor Vivian Hook, Co-Chair

Drug addiction is a chronically relapsing disorder characterized by compulsion to seek and take drugs, and the emergence of negative affective states during abstinence. These negative emotional states during withdrawal, such as anxiety and pain, are thought to contribute to compulsivity associated

with dependence, ultimately leading to drug addiction. Increased anxiety and hypersensitivity to pain are symptoms of opioid withdrawal and are considered to be factors contributing to the continued use of drugs. In the transition to opioid dependence, neuroadaptive changes occur involving recruitment of brain stress systems, such as those mediated by corticotropin-releasing factor (CRF) and norepinephrine (NE). During withdrawal, CRF/NE signaling and release are increased in the extended amygdala, specifically the central nucleus of the amygdala and bed nucleus of stria terminalis. The anxiogenic effects of CRF are mediated by CRF<sub>1</sub> receptors, which have shown to play a critical role in the negative affective states of opiate withdrawal. A CRF-NE feed-forward stress system is thought to exist between the extended amygdala and brainstem where CRF and NE affect the activation and release of the other, contributing to the presentation of negative affective states during withdrawal and consequently, compulsive drug intake. The general hypothesis was that increased anxiety and pain contribute to opioid dependence and are mediated by activation of the brain stress system. Through this thesis, anxiety- and pain-like behaviors were characterized in rats during withdrawal from acute heroin dependence and heroin self-administration, and the role of CRF/NE was explored in these behaviors as well as in compulsive heroin intake. Increased anxiety- and pain-like behavior was present during withdrawal from acute heroin injections and self-administration of heroin. CRF<sub>1</sub> receptor antagonism reversed both anxiety- and pain-like behaviors during withdrawal while noradrenergic antagonism had differing effects. The main findings suggest that CRF possibly precedes NE in

activating the brain stress system during opioid withdrawal and may be the driving force in the CRF-NE feed-forward brain stress system. In addition, CRF<sub>1</sub>R antagonism blocks heroin escalation and NE blockade decreases heroin intake, implicating the role of CRF and NE in compulsive heroin intake and opioid dependence.

## INTRODUCTION



Drug addiction is a chronically relapsing disorder characterized by a compulsion to seek and take drugs, and the emergence of a negative emotional state during abstinence. Emergence of a negative emotional state (dysphoria, anxiety, irritability) when access to the drug is prevented has been associated with the transition from drug use to addiction (Koob & Le Moal, 2005), and is thought to contribute to the compulsivity associated with dependence via the process of negative reinforcement, an increase in the probability of a response by removal of an aversive state (Koob & Le Moal, 2008). Opioid addiction is a major problem in the United States and negative emotional states during opioid withdrawal seem to play a pivotal role in the continued use of opioids. However, there is a gap in our knowledge regarding the predictive efficacy of these negative affective states for opioid dependence. A better understanding of such states may provide insights into the transition to drug addiction and possible new treatments. Therefore, the purpose of the following studies was to characterize the negative affective states associated with opioid addiction, such as heightened anxiety and increased pain sensitivity, and explore the neuropharmacological mechanisms of these behavioral responses during opioid withdrawal, ultimately leading to dependence.

Opioid addiction has been linked to dysregulation of brain emotional systems that mediate reward and stress (Koob, 2008). Opioids act on specific areas of the midbrain and ventral forebrain to produce acute positive reinforcing effects (Koob & Le Moal, 2005). Key elements of the extended amygdala not only include neurotransmitters associated with positive rewarding effects of drugs of

abuse, but also major components of the brain stress systems associated with negative reinforcement that drive dependence. In the transition to dependence, neuroadaptive changes compromise the brain reward system that involve the loss of reward neurotransmission and the recruitment of brain stress systems such as those mediated by corticotropin-releasing factor (CRF) and norepinephrine (NE) in the extended amygdala (Koob & Le Moal, 2008). The CRF system plays a major role in mediating the negative emotional states that have motivational significance in maintaining the dependent state (Zorrilla & Koob, 2010). In addition, it is thought that the neural substrates of the stress system associated with addiction overlap with substrates regulating the emotional aspects of pain and anxiety processing, including the central nucleus of the amygdala (CeA) & bed nucleus of stria terminalis (BNST) (Neugebauer & Li, 2002; Figure I-1). Therefore, characterizing the development of the negative affective states, such as anxiety and pain, during withdrawal and exploring the involvement of the brain stress system in these processes may further our understanding of the transition to opioid addiction.

Anxiety is a component of withdrawal and is considered an important factor in the continued use of drugs of abuse, as well as relapse to drugs of abuse. In animal models, a common response to acute withdrawal and protracted abstinence from all drugs of abuse is the manifestation of anxiety-like responses (Koob et al., 2009). Studies have shown that both spontaneous and antagonist-precipitated opioid withdrawal result in significant signs of anxiety-like behavior (Zhang & Schulteis, 2008). Protracted abstinence has been linked to

increases in sensitivity to anxiety-like behavior that persists after acute withdrawal in animals with a history of dependence (Koob, 2008). Similar to human alcoholics, ethanol dependent animals display enhanced anxiety-like behaviors and excessive ethanol self-administration during periods of withdrawal (Funk et al., 2006). Human alcoholics report that a negative emotional state, especially enhanced anxiety, during withdrawal is an important factor eliciting relapse and binge drinking during periods of abstinence (Hershon, 1977). There is significant co-morbidity between anxiety and drug abuse, as well as other mental disorders such as post-traumatic stress disorder (PTSD). Anxiety and stress are risk factors that predispose individuals to both the development of opioid addiction as well as relapse during withdrawal or abstinence. Spontaneous or naloxone-precipitated withdrawal results in significant signs of anxiety-like behavior (Zhang & Schulteis, 2008). Characterizing anxiety-like behavior during acute withdrawal, in addition to looking into the effects of anxiety on compulsive heroin intake will further our knowledge of treating dependence.

The acoustic startle response (ASR) is reliably elevated by a variety of anxiogenic stimuli in both humans and animals, and is blocked by anxiolytic drugs, such as benzodiazepines (Davis et al., 1993). Specifically, CRF administered intracerebroventricularly increased ASR, which was blocked by a benzodiazepine, chlordiazepoxide, and a CRF antagonist, alpha-helical CRF [9-41] without changing baseline startle levels (Swerdlow et al., 1986; 1989). The ASR can be elicited in rats and humans using identical stimulus parameters to generate equal response patterns (Koch, 1999). The results obtained in studies

with animals have been repeatedly generalized to humans, showing that the ASR is a repeatable and reliable model with face validity. In humans, aversive states and various anxiety disorders cause an enhancement of ASR; in fact, increased ASR is a diagnostic criteria for PTSD. In addition, the startle reflex is increased during withdrawal from drugs of abuse. Potentiated startle response has been observed during withdrawal from different drugs of abuse, such as ethanol, nicotine, and diazepam (Rassnick et al., 1992; Helton et al., 1993; Rasmussen et al., 1994). The increased startle response after discontinuation of drug administration may reflect the anxiety-like effects of withdrawal (Harris & Gewirtz 2004). The enhancement of ASR also occurs from the stimulation of the amygdala (Koch, 1999) and the extended amygdala is considered a key regulatory site for the effects on startle in relation to anxiety. The hypothesis was that anxiety-like behavior increases during withdrawal from acute heroin injections and escalation of heroin self-administration, and that the brain stress system, specifically the CRF and NE system, contributes to this elevated anxiety-like behavior during withdrawal from heroin. Using ASR, I looked into the effects of CRF<sub>1</sub> receptor antagonist and noradrenergic blockers on heroin-potentiated startle.

Similar to anxiety-like behavior, pain-like behavior was explored during opioid withdrawal. Opioids are the most powerful and effective drugs for the relief of pain, including emotional pain (Koob & Le Moal, 2005). Approximately one third of the adult population in the United States suffers from chronic pain (Johannes et al., 2010) and opioids continue to be the first line treatment. Many

different formulations of opioids have been proven to be effective in the treatment of a variety of chronic pain conditions, and opioids continue to be one of the best resources for analgesia and the improvement in quality of life. Although only a small percentage of chronic pain patients using opioids become addicted (Fishbain et al., 1992), recreational use and addiction to opioids continue to be a major concern. However, chronic opioid exposure for the purpose of alleviating pain often renders individuals more sensitive to nociception, a condition known as hyperalgesia (Angst & Clark, 2006). Importantly, hyperalgesia is prevalent in former opioid addicts who are maintained on methadone as a treatment of opioid dependence, suggesting that this condition emerges with extended opioid use (Compton et al., 2001). Since chronic pain is well known to cause both emotional distress and a negative emotional state, opioid-induced hyperalgesia seems to fit the diagnostic criteria as a negative affective state associated with dependence. Understanding the relationship between pain responsiveness and increase in opioid intake may further our knowledge of treatment options for dependence.

Mechanical sensitivity testing using von Frey monofilaments have been used in rats as validated pain models (Chaplan et al., 1994). Paw withdrawal thresholds (PWT) were used as the behavioral measure of pain thresholds during withdrawal. Studies from our laboratory have shown that paw withdrawal thresholds decrease during withdrawal from compulsive heroin self-administration during the two weeks of heroin escalation (Edwards et al., 2012). I tested the hypothesis that the elevation in paw withdrawal thresholds parallels the escalation of heroin self-administration, and the increase in hyperalgesia

during escalation of heroin intake also correlate with the increase in anxiety-like behavior. Both an acute withdrawal model (initiation of dependence) and a chronic withdrawal model (extended access to opioid self-administration) were used. Spontaneous and antagonist-precipitated withdrawal tests have been successfully used to measure anxiety-like behavior and hyperalgesia during acute withdrawal from opioids. The acute withdrawal model is a quick way to follow the initial development of dependence, and an easier method to test the hypothesis relevant to mechanism than self-administration procedures. In contrast, drugs that are self-administered by animals correspond well with those that have high abuse potential in humans, and intravenous drug self-administration is considered an animal model that is predictive of abuse potential (Collins et al., 1984). Successful animal models of opioid dependence have been developed using an extended access self-administration paradigm. Rodents will increase intravenous self-administration of drugs with extended access to the drugs and during withdrawal from the dependent state, as measured by increased drug intake and increased work to obtain the drug (Koob, 2008).

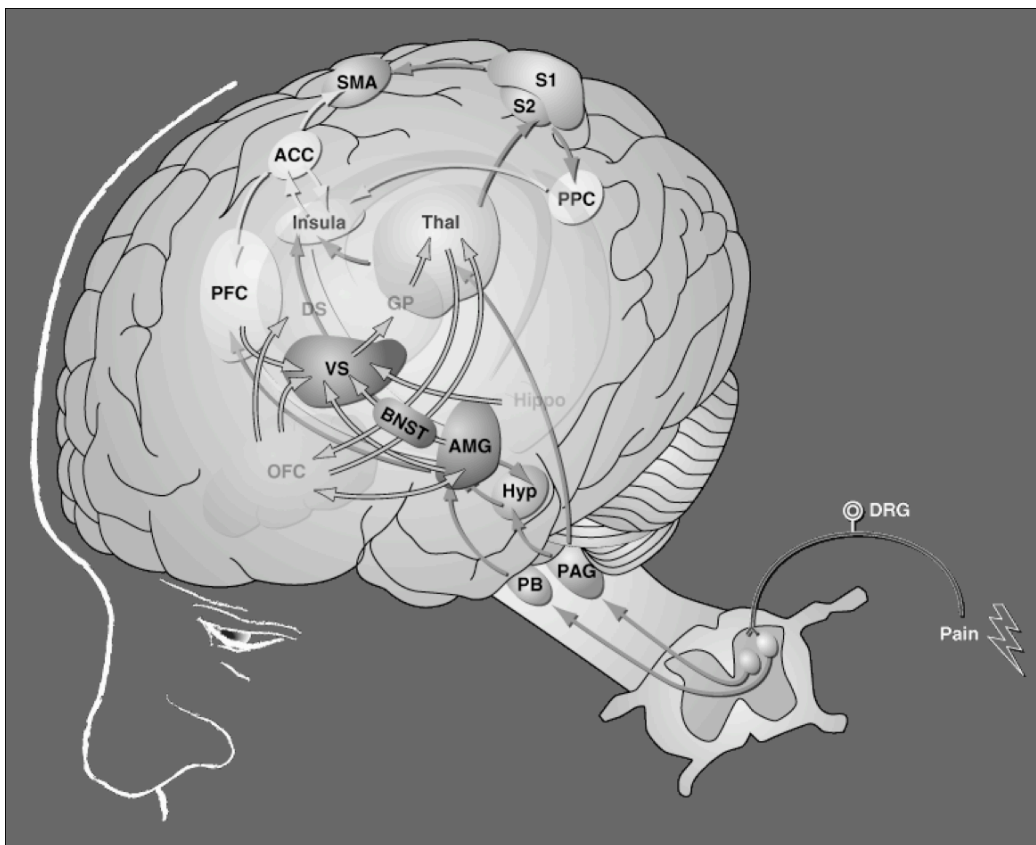
Lastly, I wanted to explore the neuropharmacological mechanisms involved in the increase in anxiety-like behavior and hyperalgesia during withdrawal from heroin, and ultimately determine the effects on heroin self-administration. Opioid-induced neuroadaptations in the brain reward and stress systems may provide insights into potential mechanisms underlying drug dependence in vulnerable individuals. Evidence points to the extended amygdala as a neural substrate of the interaction between pain and emotion, which plays a

key role in pain sensitivity and anxiety-like behavior through mechanisms that involve CRF (Shurman et al., 2010). CRF and NE are involved in stress/anxiety and are abundant in the CeA & BNST (Table I-1). Acute withdrawal from all major drugs of abuse increased CRF release in the CeA (Koob, 2008) and CRF receptor antagonists blocked excessive drug taking during drug dependence (Gilpin et al., 2008; Greenwell et al., 2009). Similarly, NE in the extended amygdala and locus coeruleus (LC) increases during acute withdrawal from drugs of abuse. Prazosin ( $\alpha_1$ -adrenergic antagonist), dose-dependently reduced heroin self-administration in rats with extended access (Greenwell et al., 2009). It is hypothesized that a CRF-NE feed-forward system exists where the CRF from the CeA/BNST activates brainstem noradrenergic activity, which in turn activates forebrain CRF (Koob, 1999). However, the order (CRF $\rightarrow$ NE, or NE $\rightarrow$ CRF) and specific locations of the activation is not known (Figure I-1). Investigating the role of CRF and NE within this feed-forward loop in relation to the negative affective states may help uncover the mechanisms of these behaviors during heroin withdrawal. Finally, pharmacological manipulations of the CRF/NE system were used to determine its effect on heroin self-administration.

In summary, the following studies were conducted to characterize the negative affective states of anxiety-like behavior and hyperalgesia in rats during withdrawal from acute heroin administration, as well as withdrawal from compulsive heroin intake using the extended access to heroin self-administration model. Further studies were aimed to explore the neurocircuitry of the brain stress system, and the role of CRF/NE on heroin self-administration. Overall,

these studies may predict sensitivity to interventions designed to reduce compulsive drug taking and develop possible treatments for opioid addiction.



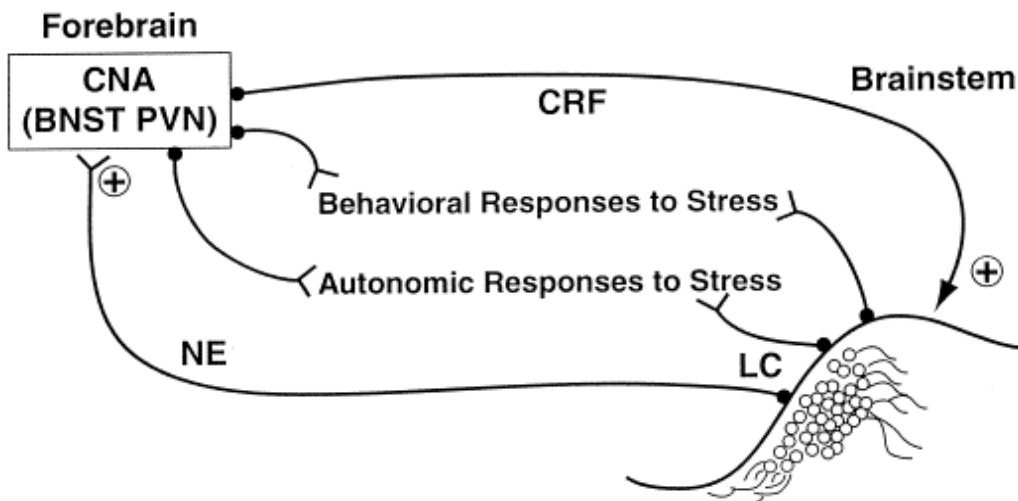


**Figure I-1.** Overlap of pain and addiction circuitry. The amygdala (AMG) receives projections from various pain pathways, including the hypothalamus (Hyp) and parabrachial nucleus (PB). The extended amygdala also plays a key role in emotionality and the negative affective states during opiate withdrawal, and its role in pain, anxiety, and addiction can be visualized through this figure.

**Table I-1.** The brain stress system is partially mediated by CRF and NE.

<b>Corticotropin-releasing factor (CRF)</b>	<b>Norepinephrine (NE)</b>
41AA peptide release from large dense-core vesicles	Neurotransmitter released from synaptic vesicles
Extrahypothalamic (independent of HPA axis), abundant in AMG/LC	Neurons originate from locus coeruleus (LC) – project to CeA/BNST
CRF1 (greater affinity), CRF2 in AMG/BNST	Adrenergic receptors in CeA/BNST
↑in CeA/BNST during withdrawal	↑in CeA/BNST during withdrawal
CRF1R antagonists block anxiogenic-like effects of acute withdrawal, block motivational signs of withdrawal, ↓heroin self-administration	Intra-CeA propranolol (nonselective $\beta$ antagonist) reversed anxiety-like behavior during withdrawal Prazosin ( $\alpha$ 1 antagonist) ↓heroin self-administration
↑NE release	↑with ICV/LC CRF

## Feed-Forward CRF-NE-CRF Stress System



**Figure I-1.** CRF-NE brain stress system. A CRF-NE feed-forward system is thought to exist where CRF from the CeA/BNST activates brainstem noradrenergic activity, which in turn activates forebrain CRF. An important question asked was whether CRF activates NE or NE activates CRF in this brain stress system.

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## **CHAPTER 1**

### **General Materials and Methods**

## **Animals**

Male Wistar rats weighing between 200-400 g were housed in groups of two to three per cage, and maintained on a 12-h light/dark cycle (lights on at 0800) with free access to food and water. The animals were allowed to acclimate to these conditions for at least 7 days in our animal facilities before behavioral testing. Animals were regularly handled for one week prior to surgery or any behavioral testing. All procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

## **Behavioral Testing**

### Acoustic Startle Response

Animals were tested for acoustic startle in the SR-LAB startle chambers for rats (San Diego Instruments, San Diego, CA, USA), which consisted of a Plexiglas cage (8 × 12.5 × 25 cm) within a ventilated, sound-attenuating chamber. Four rats were tested in four chambers simultaneously. Background noise (60 dB) and noise bursts (50 ms in duration) were presented by a speaker mounted 24 cm above the cylinder. Piezoelectric accelerometers mounted under the cylinders detected movements of the animal, which were digitized and recorded by an interface and computer assembly. Startle amplitude was defined as the maximal peak-to-peak voltage occurring during the first 200 ms after onset of the startle-eliciting stimulus. Each startle test session consisted of a 5 min



stimulus-free acclimation period with 60 dB background noise, followed by presentation of 30 startle-eliciting noise bursts (10 each at 90, 95, and 105 dB in a balanced, random order) with a 30 s fixed inter-stimulus interval. A dynamic calibration system was used to ensure comparable sensitivities across chambers. The house-light remained off throughout all testing sessions.

### Mechanical Sensitivity Testing

Up to eight rats are placed in individual plastic compartments (26 x 11 x 20cm) with stainless steel mesh floors for 30 minutes until the rats' grooming and exploratory behaviors cease. To assess the presence of mechanical hypersensitivity, the mid-plantar area of each hind paw is perpendicularly stimulated with calibrated nylon von Frey filaments (Weinstein-Semmes algometer forces) for 5 seconds using the up-down method starting with 28.84 its goo g force. A brisk withdrawal of the paw (often followed by a sustained retraction and/or licking, possibly indicative of supraspinal organization) is considered a positive response, but paw withdrawals due to locomotion or weight shifting are not counted. For quantitative assessment of mechanical sensitivity, the 50% probability withdrawal threshold (paw withdrawal threshold, PWT) will be calculated as described by Chaplan et al. (1994).

### Elevated Plus Maze Test

The plus-maze apparatus consisted of four arms elevated 50 cm above the floor, with each arm (10 cm wide, 50 cm long) positioned at 90° relative to the

adjacent arms. Two of the arms were enclosed with 40-cm high walls, and the other two arms were open. Testing was conducted in a quiet room in which the ventilation system provided approximately 65 dB background noise. The testing room was illuminated only by a dim light centered along one wall of the room such that the open arms were illuminated from the side and not down the length of the arm. To begin a test session, rats were placed in the center of the maze facing towards the enclosed arm that pointed in the direction of the light source. Two behavioral measures were recorded for each rat: 1) duration of time spent in open arms and closed arms, and 2) number of entries in each arm. This latter measure has been reported as the most reliable measure of general activity in the plus-maze. Between each trial, the maze was wiped clean with alcohol and dried with paper towels. An adjacent antechamber served as a holding room where animals were kept before drug administration, during the interval between injection and testing, and following maze testing.

### **Self-Administration Procedures**

#### Intravenous surgery and catheter patency

Rats were anesthetized with isoflurane (1.5-2%) and chronic intravenous catheters were placed in the jugular vein. Catheters consist of a 14cm length silastic tubing fitted to a guided cannula bent at a right angle and encased in dental cement anchored to a 3cm square of durable mesh. The catheter tubing is passed subcutaneously from the rat's back to the right jugular vein and 3.7cm of the silastic tubing is inserted into the vein and tied with suture. The surgeries are

performed under sterile conditions. Rats are allowed to recover for 7 days before behavioral testing. Following surgery, the catheters are flushed for 7 days with 0.2ml heparinized saline (33units/ml) that contain Timentin (20mg/0.2ml). Catheter integrity is tested whenever a rat not receiving drug pretreatments displays behavior outside baseline parameters. Rats with patent catheters that receive 0.1ml of Brevital sodium (a short-acting barbiturate) will exhibit prominent signs of anesthesia showing pronounced loss of muscle tone within 3s of IV injection. Rats with faulty catheters will be excluded from the study.

#### Intracerebral cannulations & Injections

Rats are anesthetized with isoflurane and 26 gauge, 7.5mm stainless steel guide cannulae aimed 2mm above the desired brain regions will be stereotaxically implanted bilaterally. With the incisor bar set at -3.3 from interaural 0 (flat skull), the coordinates were as follows: CeA, -2.6mm anteroposterior,  $\pm 4.2$ mm mediolateral, -5.2 dorsoventral from dura; lateral BNST, -0.35mm anteroposterior,  $\pm 3.5$ mm mediolateral ( $15^\circ$  vertical tilt), -4.5 dorsoventral from dura. The guide cannulae are secured to the skull with dental cement and anchor screws, and inserted with stylet wires to protect the brain tissue. Rats are allowed to recover at least 5d before experiments. Intracerebral injections are administered with the use of injectors (33 gauge) that projected 2mm past the guide cannula into the desired brain region. The injectors are attached to 70cm of calibrated polyethylene-20 tubing preloaded with drug solution. Injection volumes are 0.5mcl/side, infused over 1min using

microsyringes connected to injectors with tubing, controlled by an infusion pump. After drug delivery, the injectors are left in place for 60s and replaced with protective wire stylets. After a 5min preincubation period, the animals are placed into the self-administration chambers for testing.

#### Intravenous heroin self-administration

Operant chambers are located inside ventilated, sound-attenuated chambers equipped with a 1.1 watt miniature light bulb synchronized to a 12h/12h light/dark cycle (light on at 6AM). The catheter fittings on the rat's back will be connected to polyethylene tubing contained inside a protective spring suspended into the operant chamber from a liquid swivel attached to a balance arm. Drug is delivered by a syringe pump, (Razel Scientific Instruments) using a 2-rpm motor that pushes on a 30ml syringe for 4.5s to deliver a 0.1ml infusion. Each operant session is performed using two retractable levers that extend one inch into the chamber. After catheterization, male Wistar rats are trained for heroin self-administration 1h per day, on a fixed-ratio-1 schedule (every lever press is reinforced), 5 days per week. Heroin HCl is dissolved in 0.9% saline. The training dose is 60 $\mu$ g/kg/infusion, (0.1ml infusion) which is followed immediately by a cue light (above the active lever) that will remain lit for 20s signaling a time-out period. Presses during the time out period are recorded but no drug is delivered. The criterion for stable responding consists of three consecutive self-administration sessions with less than 10% variation in the total number of reinforcers. The escalation procedure begins when the rats have met

the criterion. During self-administration sessions, rats are allowed to nose-poke for food (45mg pellets, Bio-Serve), and 0.1ml water under a fixed ratio-1 schedule. The short access (ShA) group is allowed to self-administer heroin for 1h (5 PM – 6 PM), and the long-access (LgA) group for 8 - 12h (6 PM – 2 or 6 AM), 5 days a week.

## Drugs

Heroin (3,6-diacetylmorphine; International Union of Pure and Applied Chemistry: [5 $\alpha$ ,6 $\alpha$ ]-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate ester) was provided by the National Institute on Drug Abuse and was dissolved in 0.9% sterile saline. Clonidine hydrochloride ( $\alpha$ -2 agonist) was purchased from Sigma-Aldrich and dissolved in 0.9% saline and injected subcutaneously (SC) in a volume of 1 ml/kg body weight. Yohimbine hydrochloride ( $\alpha$ -2 antagonist) was purchased from Sigma-Aldrich and dissolved in sterile water and injected SC in a volume of 1 ml/kg body weight. Prazosin hydrochloride ( $\alpha$ -1 antagonist) and propranolol hydrochloride (nonselective  $\beta$  antagonist) were purchased from Sigma-Aldrich and dissolved in 0.9% saline and injected intraperitoneously (IP) in a volume of 1 ml/kg body weight. Chlordiazepoxide hydrochloride was purchased from Sigma-Aldrich and dissolved in 0.9% sterile saline, injected IP in a volume of 1ml/kg body weight. The CRF 1 receptor antagonist N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo[1,5-a]pyrimidin-7-amine (MPZP) was prepared for systemic administration by first dissolving in 1 M HCl (10% final

volume) and diluted with 25% (w/v) hydroxypropyl  $\beta$ -cyclodextrin (HBC, Cargill, Cedar Rapids, IA) (80% final volume), then back-titrated with descending concentrations of NaOH (2, 1, 0.1 M) (10% final volume), resulting in a final suspension of 10 mg/ml MPZP in 20% HBC (pH 4.5). Animals were administered MPZP in a volume of injection of 2 ml/kg SC. For the 0 mg/kg dose of MPZP, animals were given 2 ml 20% HBC vehicle/kg body weight.

### **Statistical Analysis**

All data are expressed as means and standard errors of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) with treatment (repeated saline, repeated heroin) as a between-subjects factor or using repeated-measure two-way ANOVA with group as a between-subjects factor and drug and intensity (90, 95, and 105 dB) as within-subjects factors. Fisher's Least Significant Difference (LSD) or Dunnett's test was used for *post hoc* comparisons when appropriate. All statistical analyses were performed with Statistica 10, and p-values less than 0.05 were considered statistically significant.

## **CHAPTER 2**

### **Validation Studies for the Acoustic Startle Response Model**

## INTRODUCTION

The acoustic startle response (ASR) has been used as a measure of anxiety-like behavior and ASR can be elicited in rats and humans using identical stimulus parameters to generate equal response patterns (Koch, 1999). The results obtained in studies with animals have been repeatedly generalized to humans, showing that the ASR is a repeatable and reliable model with face validity. In humans, aversive states and various anxiety disorders cause an enhancement of ASR; in fact, increased ASR is a diagnostic criteria for posttraumatic stress disorder (PTSD). ASR is reliably elevated by a variety of anxiogenic stimuli in both humans and animals, and is blocked by anxiolytic drugs, such as benzodiazepines (Davis et al., 1993). Yohimbine ( $\alpha$ -2 antagonist) has been shown to elevate startle in rodents (Davis and Astrachan, 1981) and central descending NE neurons are necessary for its excitatory effects on startle (Kehne and Davis, 1985). In addition, CRF administered intracerebroventricularly increased ASR, which was blocked by a benzodiazepine, chlordiazepoxide, and a CRF antagonist, alpha-helical CRF [9-41], without changing baseline startle levels (Swerdlow et al., 1986; 1989). The enhancement of ASR also occurs from the stimulation of the amygdala (Koch, 1999) and the extended amygdala is considered a key regulatory site for the effects on startle in relation to anxiety.

In addition, the startle reflex is increased during withdrawal from drugs of abuse. Potentiated startle response has been observed during withdrawal from different drugs of abuse, such as ethanol, nicotine, diazepam, and morphine (Rassnick et al., 1992; Helton et al., 1993; Rasmussen et al., 199; Harris &



Gewirtz, 2004). The increased startle response after discontinuation of drug administration may reflect the anxiety-like effects of withdrawal (Harris & Gewirtz, 2004). Therefore, ASR was used as the behavioral measure of anxiety-like behavior during acute withdrawal from both heroin administration and heroin self-administration. In order to develop a reliable and repeatable measure, I performed several validation studies that are described below.

## **MATERIALS AND METHODS**

Details of the Acoustic Startle Response procedure, drugs and statistical analyses used are described in Chapter 1: Materials and Methods.

## **RESULTS**

I wanted to confirm the potentiation of startle by yohimbine at different doses. Figure 2-1A shows the robust potentiation of startle with yohimbine at 1.25 mg/kg (SC). ANOVA revealed that there was an interaction between group and intensity ( $F_{(2, 28)} = 9.11, p < 0.001$ ). Post-hoc analyses showed that yohimbine-treated rats' ASR was significantly different from vehicle-treated rats at 95 dB ( $p < 0.005$ ), and 105 dB ( $p < 0.0001$ ). There was an overall group effect ( $F_{(1, 14)} = 16.60, p < 0.005$ ), and an intensity effect ( $F_{(2, 28)} = 63.75, p < 0.0001$ ). Figure 2-1B shows the potentiation of ASR by 1.25 mg/kg yohimbine was reversed by chlordiazepoxide at 5 mg/kg (IP). ANOVA revealed an interaction between group and intensity ( $F_{(2, 26)} = 11.04, p < 0.0005$ ). Post-hoc analyses revealed that at 105 dB, yohimbine-vehicle treated rats showed a significantly

higher ASR than yohimbine-CDP treated rats ( $p < 0.0001$ ). A group effect ( $F_{(1, 13)} = 6.10$ ,  $p < 0.05$ ) and an intensity effect was present ( $F_{(2, 26)} = 44.38$ ,  $p < 0.0001$ ).

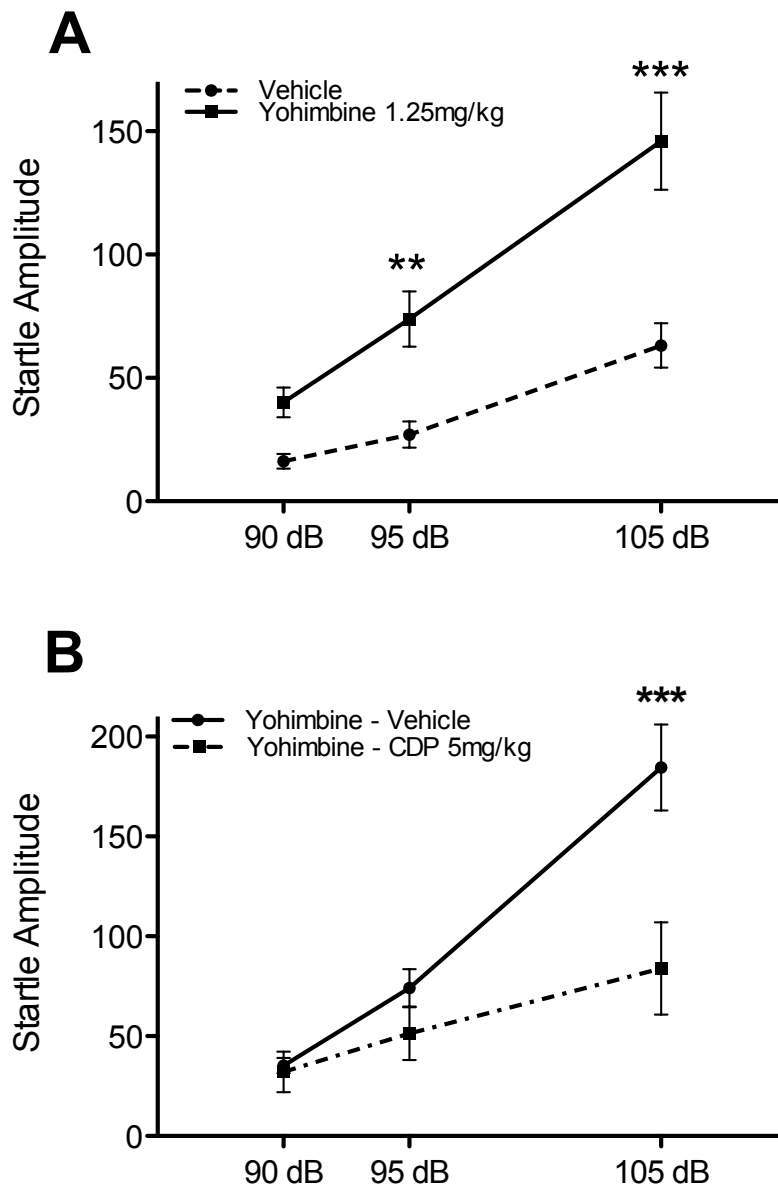
Figure 2-2A represents the repeatability of ASR over different days of testing. Yohimbine was tested in the same rats over 2 different days and showed similar magnitude of potentiation compared to vehicle treated rats. ANOVA for repeated measures revealed a group  $\times$  intensity effect:  $F_{(2, 28)} = 5.17$ ,  $p < 0.05$ . Post-hoc analysis indicated that yohimbine potentiated startle significantly at 95 dB ( $p < 0.01$ ) and 105 dB ( $p < 0.0001$ ). A group effect ( $F_{(1, 14)} = 13.59$ ,  $p < 0.005$ ) and an intensity effect ( $F_{(2, 28)} = 66.68$ ,  $p < 0.0001$ ) was also observed. A day effect was not seen ( $F_{(1, 14)} = 1.79$ ,  $p > 0.2$ ). Figure 2-2B shows the strong correlation between two days of testing. Pearson correlation indicated a significant positive correlation for startle amplitudes between day 1 and day 2 ( $r = 0.88$ ;  $p < 0.0001$ ) confirming the repeatability of the acoustic startle response.

Figure 2-3 represents the dose-dependent potentiation of startle by yohimbine (0.3125 mg/kg, 0.625 mg/kg, 1.25 mg/kg SC). ANOVA revealed a dose effect of yohimbine ( $F_{(2, 21)} = 7.38$ ,  $p < 0.005$ ). Post-hoc analyses showed that yohimbine 1.25mg/kg produced significantly higher startle amplitude compared to 0.625mg/kg ( $p < 0.05$ ) and 0.3125mg/kg ( $p < 0.005$ ).

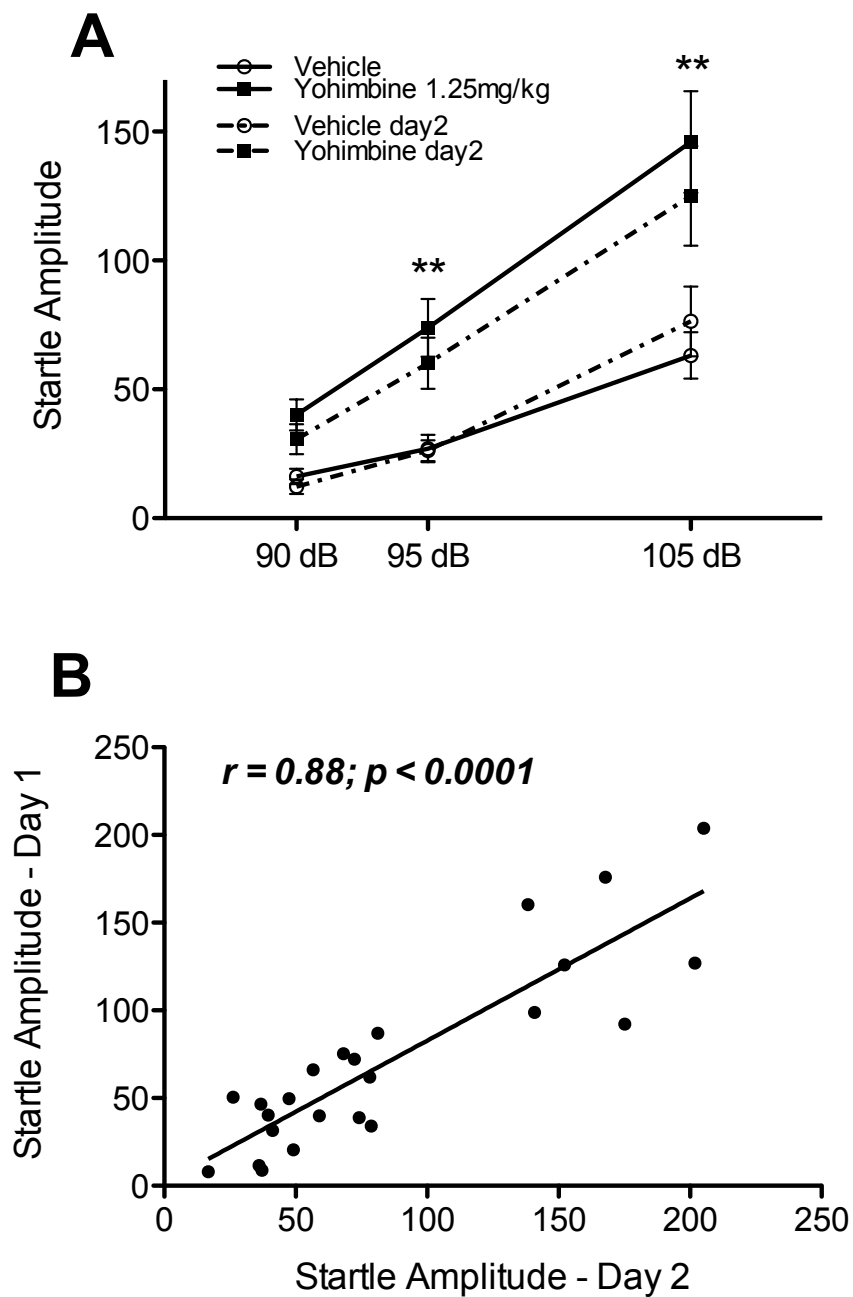
ASR has been shown to increase during morphine withdrawal (Harris & Gewirtz, 2004) and we wanted to be able to replicate these findings. Figure 2-4 shows the effect of morphine withdrawal-potentiated startle at four hours post morphine (10 mg/kg, SC). The ANOVA revealed that at 105 dB, a group  $\times$  days interaction was observed ( $F_{(4, 56)} = 3.74$ ,  $p < 0.01$ ). Post-hoc analyses indicated

that morphine treated rats on days 1, 2, and 4 were significantly higher than saline-treated rats ( $p < 0.05$ ).

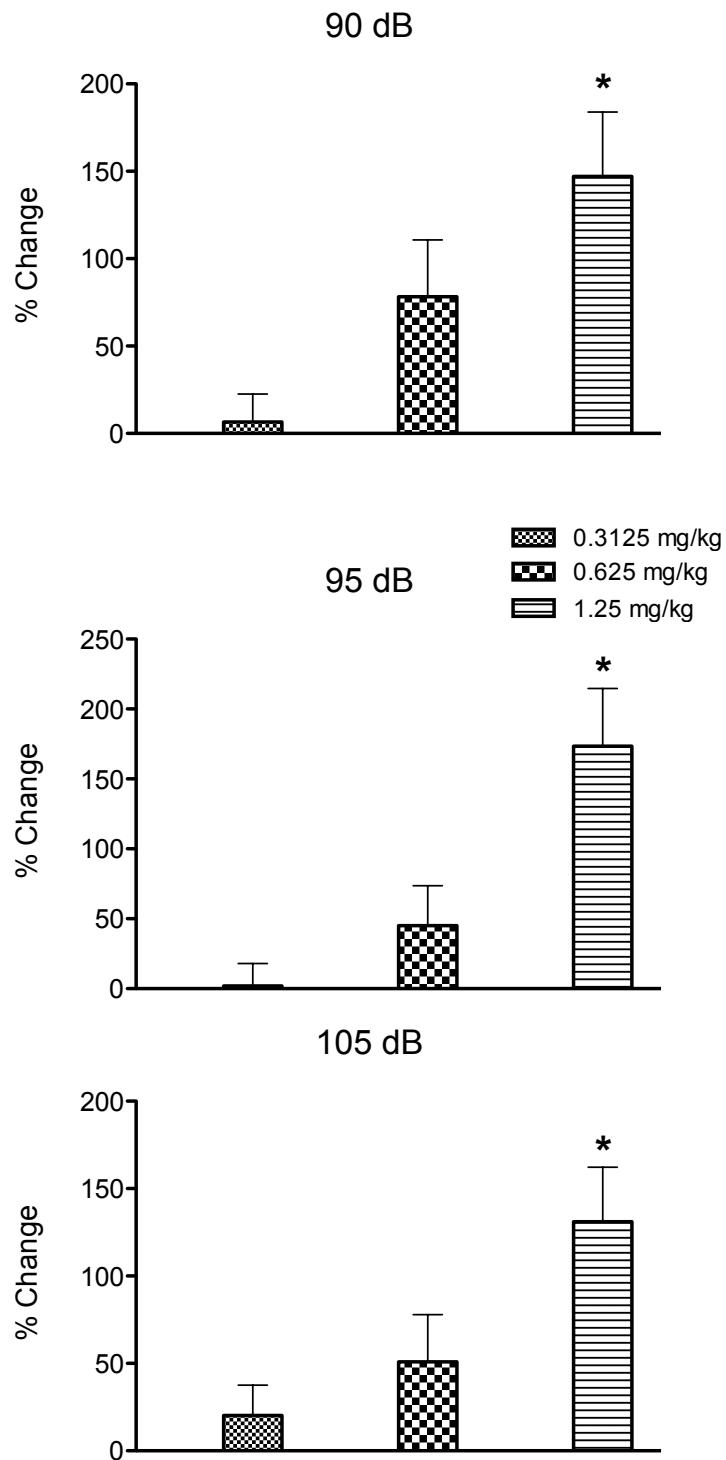
Finally, I wanted to use the ASR to confirm that intracerebroventricular CRF potentiates startle as previously shown. Figure 2-5A shows the effect of ICV CRF on ASR. ANOVA revealed an interaction between group and intensity ( $F_{(2, 36)} = 6.67, p < 0.005$ ). Post-hoc analysis revealed that at 105 dB, rats treated with ICV CRF showed a significantly higher ASR compared to rats treated with ICV saline ( $p < 0.0001$ ). There was also a group effect ( $F_{(1, 18)} = 12.0, p < 0.005$ ) and an intensity effect ( $F_{(2, 36)} = 41.8, p < 0.0001$ ). Figure 2-5B shows the locomotor activity difference between CRF-treated rats and saline-treated rats. ANOVA revealed a group effect ( $F_{(1, 13)} = 12.5, p < 0.005$ ) and time effect ( $F_{(12, 156)} = 6.78, p < 0.0001$ ) showing CRF-treated rats with higher locomotor activity compared to saline-treated rats.



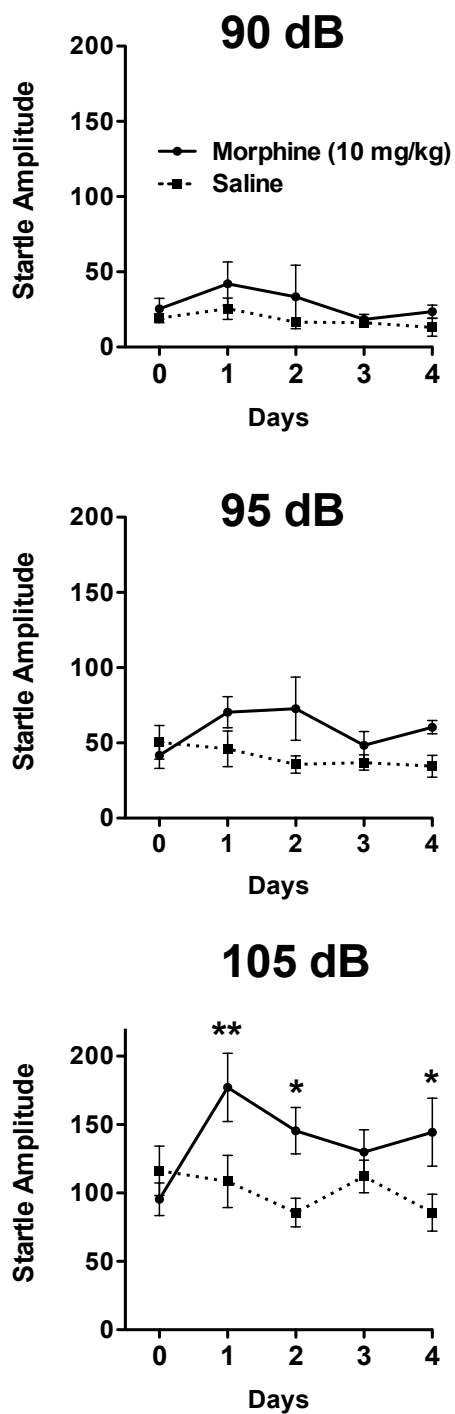
**Figure 2-1.** Yohimbine-potentiated startle. (a) Yohimbine (1.25 mg/kg SC)-treated rats show significantly potentiated startle at 95 dB and 105 dB (\*\* p < 0.005, \*\*\* p < 0.0001) compared to vehicle-treated rats. (b) Chlordiazepoxide (5 mg/kg IP) blocks yohimbine-potentiated startle (\*\*\*) p < 0.0001).



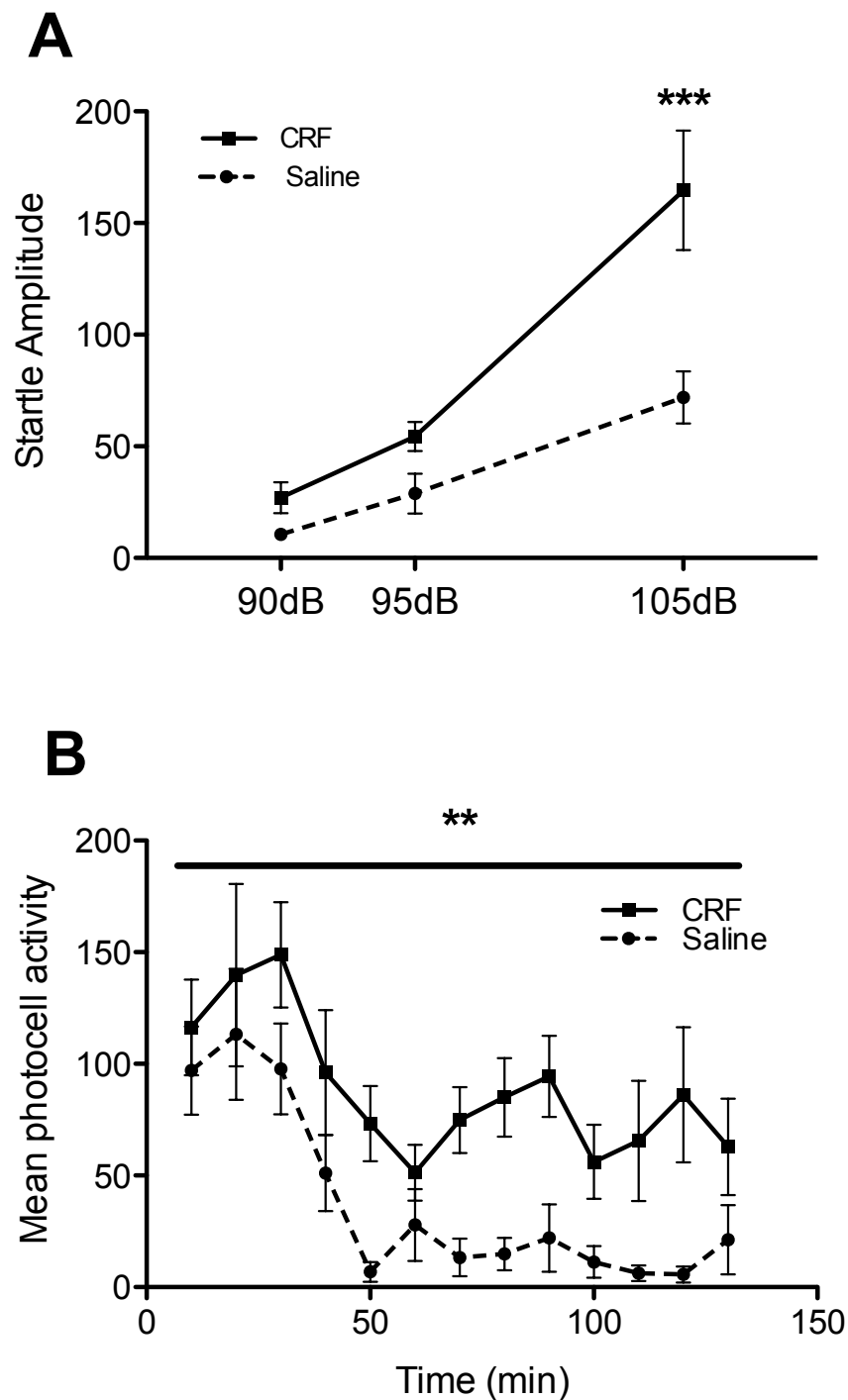
**Figure 2-2.** Yohimbine-potentiated startle over two days of testing. (a) Yohimbine (1.25 mg/kg SC)-treated rats showed significantly potentiated startle at 95 dB and 105 dB (\*\*  $p < 0.01$ ) compared to vehicle-treated rats. (b) Positive correlation between two days of testing, showing the repeatability of ASR.



**Figure 2-3.** Yohimbine dose-dependently increases ASR. Yohimbine (1.25 mg/kg SC) produced significantly higher startle amplitude compared to 0.625 mg/kg and 0.3125 mg/kg (\*  $p < 0.05$ ).



**Figure 2-4.** Morphine withdrawal potentiates startle. ASR is potentiated 4 h after morphine (10 mg/kg SC) administration consistently over four days. At 105 dB, ASR is significantly potentiated on day 1, 2 & 4 (\*\*  $p < 0.005$ , \*  $p < 0.05$ ).



**Figure 2-5.** CRF potentiates startle. (a) ICV CRF significantly potentiated startle at 105 dB (\*\*\*)  $p < 0.0001$ ). (b) CRF-treated rats showed higher locomotor activity compared to saline-treated rats (\*\*  $p < 0.005$ ).



## DISCUSSION

Through the series of validation studies, we confirmed the acoustic startle response as a repeatable measure of anxiety-like behavior in rats. Yohimbine ( $\alpha 2$  antagonist) dose-dependently increased ASR and the 1.25 mg/kg (SC) dose elevated startle over multiple testing days with significant correlation. Chlordiazepoxide (5 mg/kg IP) blocked the yohimbine-potentiated startle. Previously, yohimbine was shown to increase startle (Davis and Astrachan, 1981; Kehne and Davis, 1985), and benzodiazepines blocked the increase in startle from anxiogenic stimuli (Davis et al., 1993). ASR was elevated during withdrawal from morphine (10 mg/kg SC) over four days while the saline treated groups did not show an increase in startle. We observed that for the saline treated rats, testing over multiple days did not result in habituation or a significant lowering of the startle amplitude. Intracerebroventricular CRF also significantly elevated startle similar to what has been shown previously (Swerdlow et al., 1986). In order to confirm that the elevation of startle was from CRF, we tested locomotor activity on the same rats. Rats that were administered ICV CRF showed overall higher photocell activity compared to saline administered rats. Overall, we were able to replicate previous findings and validate the ASR as a consistent measure of anxiety-like behavior in rats. For the studies listed in the following chapters, withdrawal-potentiated startle was used as the anxiety-like behavioral measure.

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## **CHAPTER 3**

**Corticotropin-releasing factor (CRF) and  $\alpha$ -2 adrenergic receptors mediate  
heroin withdrawal-potentiated startle in rats**

**ABSTRACT**

Anxiety is one of the early symptoms of opioid withdrawal and contributes to continued drug use and relapse. The acoustic startle response (ASR) is a component of anxiety that has been shown to increase during opioid withdrawal in both humans and animals. We investigated the role of corticotropin-releasing factor (CRF) and norepinephrine (NE), two key mediators of the brain stress system, on acute heroin withdrawal-potentiated ASR. Rats injected with heroin (2 mg/kg, SC) displayed an increased ASR when tested 4 h after heroin treatment. A similar increase in the ASR was found in rats 10-20 h into withdrawal from extended access (12 h) to intravenous heroin self-administration, a model that captures several aspects of heroin addiction in humans. Both the  $\alpha_2$  adrenergic receptor agonist clonidine (10  $\mu$ g/kg, SC) and CRF<sub>1</sub> receptor antagonist *N,N*-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo[1,5-*a*]pyrimidin-7-amine (MPZP; 20 mg/kg, SC) blocked heroin withdrawal-potentiated startle. To investigate the relationship between CRF<sub>1</sub> and  $\alpha_2$  adrenergic receptors in the potentiation of the ASR, we tested the effect of MPZP on yohimbine (1.25 mg/kg, SC)- potentiated startle and clonidine on CRF (2  $\mu$ g, ICV)-potentiated startle. Clonidine blocked CRF-potentiated startle, whereas MPZP partially attenuated but did not reverse yohimbine-potentiated startle, suggesting that CRF may drive NE release to potentiate startle. These results suggest that CRF<sub>1</sub> and  $\alpha_2$  receptors play an important role in the heightened anxiety-like behavior observed during acute withdrawal from heroin, possibly via CRF inducing the release of NE in stress-related brain regions.

## INTRODUCTION

Drug addiction is a chronically relapsing disorder characterized by a compulsion to seek and take drugs and the emergence of a negative emotional state during abstinence (Koob and Le Moal, 2005). A negative affective state is defined as a dysphoric state accompanied by depressive-like and anxiety-like symptoms (Koob and Le Moal, 2008) and is thought to contribute to the compulsivity associated with dependence via the process of negative reinforcement (i.e., an increase in the probability of a response by removal of an aversive state). In human opioid addicts, anxiety is a component of the initial stages of withdrawal and the development of anxiety disorders may be a consequence of ongoing opiate addiction (Fatséas *et al*, 2010). Studies in laboratory animals have also shown that both spontaneous and antagonist-precipitated opioid withdrawal results in significant signs of anxiety-like behavior (Harris and Aston-Jones, 1993; Schulteis *et al.*, 1998; Zhang and Schulteis, 2008) and neuroadaptations in anxiety-related brain regions (Edwards *et al.*, 2009). Thus, anxiety appears to be an important factor in the continued use of drugs and relapse to drug seeking and taking. However, there is a gap in our knowledge about the neuropharmacological mechanisms that underlie anxiety-like behavior during opioid withdrawal.

Opioid addiction has been linked to dysregulation of brain emotional systems that mediate reward and stress (Koob, 2008). Opioids act on specific areas of the midbrain and ventral forebrain to produce acute positive reinforcing effects (Koob and Le Moal, 2005). In the transition to dependence, neuroadaptive

changes compromise the brain reward system, involving the loss of reward neurotransmission and recruitment of brain stress systems, such as those mediated by corticotropin-releasing factor (CRF) and norepinephrine (NE) in the extended amygdala (Koob and Le Moal, 2008). Extracellular CRF in the extended amygdala is increased during acute withdrawal from drugs of abuse, and CRF receptor antagonists block excessive drug taking during dependence (Funk, 2007; Specio, 2008; Greenwell, 2009). The anxiogenic effects of CRF have been reported to be mediated by CRF<sub>1</sub> receptors, whereas CRF<sub>2</sub> receptors have been shown to modulate CRF effects on feeding behavior with a lesser role in anxiety-like behavior (Spina 1996; Smith, 1998; Risbrough, 2003). CRF<sub>1</sub> receptor-deficient animals did not show opiate withdrawal-induced place aversions, demonstrating a critical role of CRF<sub>1</sub> receptors in the negative affective states of opiate withdrawal (Contarino and Papaleo, 2005). Similarly, NE in the extended amygdala and locus coeruleus increases during acute withdrawal from drugs of abuse. Specifically, hyperactivity of brain NE has been implicated in mechanisms of opiate withdrawal in the extended amygdala, which is also accompanied by increased signaling of CRF (Maldonado, 1997; Aston-Jones *et al.*, 1999; Smith and Aston-Jones, 2008). It has been hypothesized that a CRF-NE feed-forward system exists, in which CRF from the central nucleus of the amygdala (CeA)/bed nucleus of stria terminalis (BNST) activates brainstem noradrenergic activity, which then activates forebrain CRF (Koob, 1999). However, whether CRF or NE is the driving force in such a feed-forward system in opioid withdrawal-induced anxiety is not yet known.

The acoustic startle response (ASR) is reliably elevated by anxiogenic-like stimuli in both humans and laboratory animals and is blocked by anxiolytic-like agents (Davis *et al*, 1993). The  $\alpha_2$  receptor antagonist yohimbine is an anxiogenic-like agent that increases startle, and its effect is likely mediated through central descending NE neurons (Kehne and Davis, 1985). Intracerebroventricular administration of CRF also increased ASR, which was blocked by the benzodiazepine chlordiazepoxide and the CRF antagonist  $\alpha$ -helical CRF<sub>9-41</sub> (Swerdlow *et al*, 1986; 1989). Potentiated startle responses have been observed during withdrawal from different drugs of abuse, including ethanol, nicotine, diazepam, and morphine (Rassnick *et al*, 1992; Helton *et al*, 1993; Rasmussen *et al*, 1994; Harris and Gewirtz, 2004). Therefore, the increased startle response after the discontinuation of drug administration may reflect the anxiety-like effects of withdrawal (Harris and Gewirtz, 2004).

In the present study, we tested the hypothesis that the brain stress system, specifically the CRF and NE systems, contributes to elevated anxiety-like behavior during withdrawal from heroin and that CRF is the driving force of the CRF-NE brain stress system. Using the ASR, we characterized anxiety-like behavior during withdrawal from acute heroin exposure and heroin self-administration. We then tested the effects of CRF<sub>1</sub> receptor antagonist MPZP and  $\alpha_2$  adrenergic receptor agonist clonidine on heroin-potentiated startle. Finally, we tested the effect of MPZP on yohimbine-potentiated startle and the effect of clonidine on CRF-potentiated startle.

## **MATERIALS AND METHODS**

### **Animals**

Male Wistar rats ( $n = 129$ ), weighing 200-400 g, were housed in groups of two to three per cage and maintained on a 12 h/12 h light/dark cycle (lights on at 8:00 AM) with free access to food and water. The animals were allowed to acclimate to these conditions for at least 7 days in our animal facilities before behavioral testing. The animals were regularly handled for 1 week prior to surgery and any behavioral testing. All of the procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

### **Acoustic startle test**

The animals were tested for acoustic startle in SR-LAB startle chambers for rats (San Diego Instruments, San Diego, CA, USA), which consisted of a Plexiglas cage ( $8 \times 12.5 \times 25$  cm) within a ventilated, sound-attenuating chamber. Four rats were tested in four chambers simultaneously. Background noise (60 dB) and noise bursts (50 ms duration) were presented by a speaker mounted 24 cm above the cylinder. Piezoelectric accelerometers mounted under the cylinders detected the movements of the animal, which were digitized and recorded by an interface and computer assembly. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting stimulus. Each startle test session consisted of a



5 min stimulus-free acclimation period with 60 dB background noise, followed by presentation of 30 startle-eliciting noise bursts (10 each at 90, 95, and 105 dB in a balanced, random order) with a 30 s fixed interstimulus interval. A dynamic calibration system was used to ensure comparable sensitivities across chambers. The house light remained off throughout the test sessions.

## Drugs

Heroin (3,6-diacetylmorphine; International Union of Pure and Applied Chemistry: [5 $\alpha$ ,6 $\alpha$ ]-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate ester) was provided by the National Institute on Drug Abuse, dissolved in 0.9% sterile saline, and injected subcutaneously (SC). The  $\alpha_2$  receptor agonist clonidine hydrochloride was purchased from Sigma-Aldrich, dissolved in 0.9% saline, and injected subcutaneously in a volume of 1 ml/kg body weight. The  $\alpha_2$  receptor antagonist yohimbine hydrochloride was purchased from Sigma-Aldrich, dissolved in sterile water, and injected subcutaneously in a volume of 1 ml/kg body weight. The CRF<sub>1</sub> receptor antagonist *N,N*-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo[1,5-*a*] pyrimidin-7-amine (MPZP) was prepared for systemic administration by dissolving in 1 M HCl (10% final volume), diluting with 25% (w/v) hydroxypropyl  $\beta$ -cyclodextrin (HBC; Cargill, Cedar Rapids, IA; 80% final volume), and back-titrating with descending concentrations of NaOH (2, 1, and 0.1 M; 10% final volume), resulting in a final suspension of 10 mg/ml MPZP in 20% HBC (pH 4.5). The animals were subcutaneously administered MPZP in a volume of 2 ml/kg. For the 0 mg/kg

dose of MPZP, the animals were given 2 ml of 20% HBC vehicle/kg body weight.

### **Heroin self-administration**

The surgery and self-administration procedures have been reported in detail previously (Vendruscolo *et al*, 2011). Briefly, the rats were anesthetized with isoflurane (1.5-2%), and chronic intravenous catheters were implanted in the jugular vein. The Rats were allowed to recover for 7 days before behavioral testing.

Operant chambers (Med Associates, St Albans, VT) were located inside ventilated, sound-attenuated chambers equipped with a 1.1-watt miniature light bulb synchronized to a 12 h/12 h light/dark cycle (lights on at 6:00 AM). The catheter fittings on the rat's back were connected to polyethylene tubing contained inside a protective metal spring suspended into the operant chamber from a liquid swivel attached to a balance arm. Drug was delivered by activating a syringe pump (Razel Scientific Instruments) with a 2-rotations-per-minute motor that pushed on a 30 ml syringe for 4.5 s to deliver a 0.1 ml infusion. Each operant session was performed using two retractable levers that extended 1 inch into the chamber.

The rats were trained to lever press for heroin (60 µg/kg/infusion) 1 h per day on a fixed-ratio (FR) 1 schedule (i.e., every lever press was reinforced) for 5 days per week. Drug infusions were paired with a 20 s cue light above the active lever that signaled a timeout period. Presses during the timeout period were recorded, but no drug was delivered. Once stable lever pressing was achieved

(i.e., three consecutive self-administration sessions with less than 10% variation in the total number of reinforcers), the animals were split into two groups that were matched for responding: long-access (LgA; 12 h) and short-access (ShA; 1 h) to heroin self-administration. During the self-administration sessions, the rats are allowed to nosepoke for food (45 mg pellets, Bio-Serve) on an FR3 schedule and water on an FR1 schedule. Rats were tested for ASR 10-20 h into withdrawal from heroin self-administration.

### **Intracerebroventricular CRF infusion**

The rats were implanted with indwelling cannulae directed unilaterally at the lateral ventricle. The rats were anesthetized with isoflurane (1.5-2%) and secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and a 22-gauge stainless steel cannula (Plastics One, Roanoke, VA) was aimed 1 mm above the lateral ventricle and secured to the skull with four stainless steel screws and Silux dental cement. The stereotaxic coordinates were the following: anterior/posterior, -0.6 mm; medial/lateral,  $\pm$  2.0 mm relative to bregma; dorsal/ventral, -3.2 mm from skull surface. A 7 mm dummy stylet (Plastics One, Roanoke, VA) filled the cannula and maintained patency. The animals were allowed 1 week to recover from surgery before testing. Intracerebroventricular (ICV) rat/human CRF (2  $\mu$ g/4  $\mu$ l) was injected using a Hamilton microsyringe and a 30-gauge stainless steel injector attached to polyethylene 20 tubing. The injector projected 1 mm beyond the end of the cannula.

## Pharmacological testing

All of the pretreatment times were derived from previous studies. For pharmacological testing that involved acute heroin withdrawal-potentiated startle, the rats were injected with heroin (2 mg/kg) or saline 4 h prior to ASR testing and then administered a pretreatment drug at different time-points prior to ASR testing. Clonidine (10 µg/kg) and MPZP (20 mg/kg) were administered 60 min prior to ASR testing. Yohimbine (1.25 mg/kg) was injected 30 min prior to testing. All of the pharmacological testing was performed using a Latin-square design, in which the pretreatment groups were switched on the following test day (i.e., 2-3 days after the preceding test), with the exception of the experiment that involving ICV CRF, in which all of the rats were tested once.

## Statistical analysis

All of the data are expressed as means and standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA), with group (repeated saline, repeated heroin) as the between-subjects factor, or two-way repeated-measures ANOVA, with group (saline vs. heroin, vehicle vs. yohimbine, saline vs. CRF) as the between-subjects factor and treatment (clonidine and MPZP vs. vehicle) and intensity (90, 95, and 105 dB) as the within-subjects factors. Fisher's Least Significant Difference (LSD) *post hoc* test was used when appropriate. All of the statistical analyses were performed using Statistica 10. Values of  $p < 0.05$  were considered statistically significant.

## RESULTS

### **Withdrawal from acute heroin administration or extended access to heroin self-administration potentiated startle**

Figure 3-1 illustrates the acoustic startle response in rats during withdrawal from a heroin injection (4 h into withdrawal) or self-administration (10-20 h into withdrawal). For acute heroin injection (Fig. 3-1a), the ANOVA revealed a significant effect of heroin ( $F_{1,28} = 7.83, p < 0.01$ ), with heroin-treated rats showing higher startle amplitudes compared with saline-treated rats. An effect of intensity was also found ( $F_{1,56} = 133.9, p < 0.0001$ ), showing startle amplitudes at 90 dB < 95 dB < 105 dB. Similarly, Fig. 1b shows that rats with extended access (12 h) to heroin displayed a significant increase in the startle response during withdrawal compared with rats with limited access (1 h) to heroin at 105 dB (treatment  $\times$  intensity interaction:  $F_{2,32} = 5.51, p < 0.01$ ; LSD *post hoc* test,  $p < 0.005$ ). An effect of intensity was also present ( $F_{2,32} = 46.19, p < 0.0001$ ). ASR was evaluated after day 1 of self-administration. The average number of lever press on day 1 for the ShA group was  $5.3 \pm 0.9$ , and LgA group was  $42.7 \pm 9.9$ .

### **MPZP blocked heroin withdrawal-potentiated startle**

Figure 3-2 shows the effect of MPZP (20 mg/kg, SC) on acute heroin withdrawal-potentiated startle. The ANOVA revealed a group (heroin)  $\times$  treatment interaction ( $F_{1,23} = 4.5, p < 0.05$ ). *Post hoc* comparisons showed that animals treated with heroin + vehicle displayed higher startle amplitudes at 105 dB compared with all of the other groups ( $p < 0.01$ ). MPZP blocked heroin-

potentiated startle, in which heroin-MPZP-treated rats displayed significantly lower startle amplitudes compared with heroin-vehicle-treated rats ( $p < 0.01$ ). The ASR was not different between heroin-MPZP- and saline-vehicle-treated rats, and MPZP alone did not alter the ASR. An effect of intensity was found ( $F_{1,56} = 133.9, p < 0.0001$ ), showing startle amplitudes at 90 dB < 95 dB < 105 dB.

### **Clonidine blocked heroin withdrawal-potentiated startle**

Figure 3-3 displays the effect of clonidine (10  $\mu\text{g}/\text{kg}$ , SC) on acute heroin withdrawal-potentiated startle. The ANOVA revealed a group (heroin)  $\times$  treatment  $\times$  intensity interaction ( $F_{2,26} = 5.6, p < 0.01$ ). The *post hoc* comparisons indicated that heroin-treated rats displayed higher startle amplitudes at 105 dB compared with saline-treated rats ( $p < 0.0005$ ). Importantly, heroin-treated rats that received clonidine displayed significantly lower startle amplitudes at 105 dB than heroin-treated rats that received saline ( $p < 0.0001$ ). At this dose, clonidine alone did not produce an effect on the startle response. An effect of intensity was found ( $F_{2,26} = 161.4, p < 0.0001$ ), showing startle amplitudes at 90 dB < 95 dB < 105 dB.

### **MPZP did not block yohimbine-potentiated startle**

In a preliminary study, we observed a dose-dependent potentiation of startle induced by yohimbine. Table 3-1 shows the dose-response effect of yohimbine on the ASR. The ANOVA revealed a significant yohimbine dose  $\times$  intensity and ( $F_{6,42} = 9.2, p < 0.0001$ ). Subsequent analyses indicated that 1.25

mg/kg yohimbine significantly increased the ASR compared with all of the other doses ( $p < 0.0001$ ) at 105 dB. Based on our results and previous findings (Kehne and Davis 1985), we chose a 1.25 mg/kg dose of yohimbine to determine whether MPZP, a CRF<sub>1</sub> receptor antagonist, blocks the startle potentiation induced by yohimbine. Figure 3-4 shows the effect of systemic MPZP (20 mg/kg, SC) on yohimbine-potentiated startle. The ANOVA revealed a significant group  $\times$  intensity interaction ( $F_{2,40} = 3.78, p < 0.05$ ). The *post hoc* comparisons indicated that yohimbine produced a significant increase in startle at 105 dB ( $p < 0.005$ ). MPZP partially attenuated but did not completely block yohimbine-potentiated startle (Yohimbine  $\times$  MPZP interaction:  $F_{1,20} = 0.14, p = 0.72$ ). MPZP alone (MPZP-Vehicle vs. Vehicle-Vehicle) did not have a significant effect ( $F_{1,20} = 3.42, p = 0.079$ ). An effect of intensity was found ( $F_{2,40} = 83.38, p < 0.0001$ ), showing startle amplitudes at 90 dB  $<$  95 dB  $<$  105 dB.

### **Clonidine blocked CRF-potentiated startle**

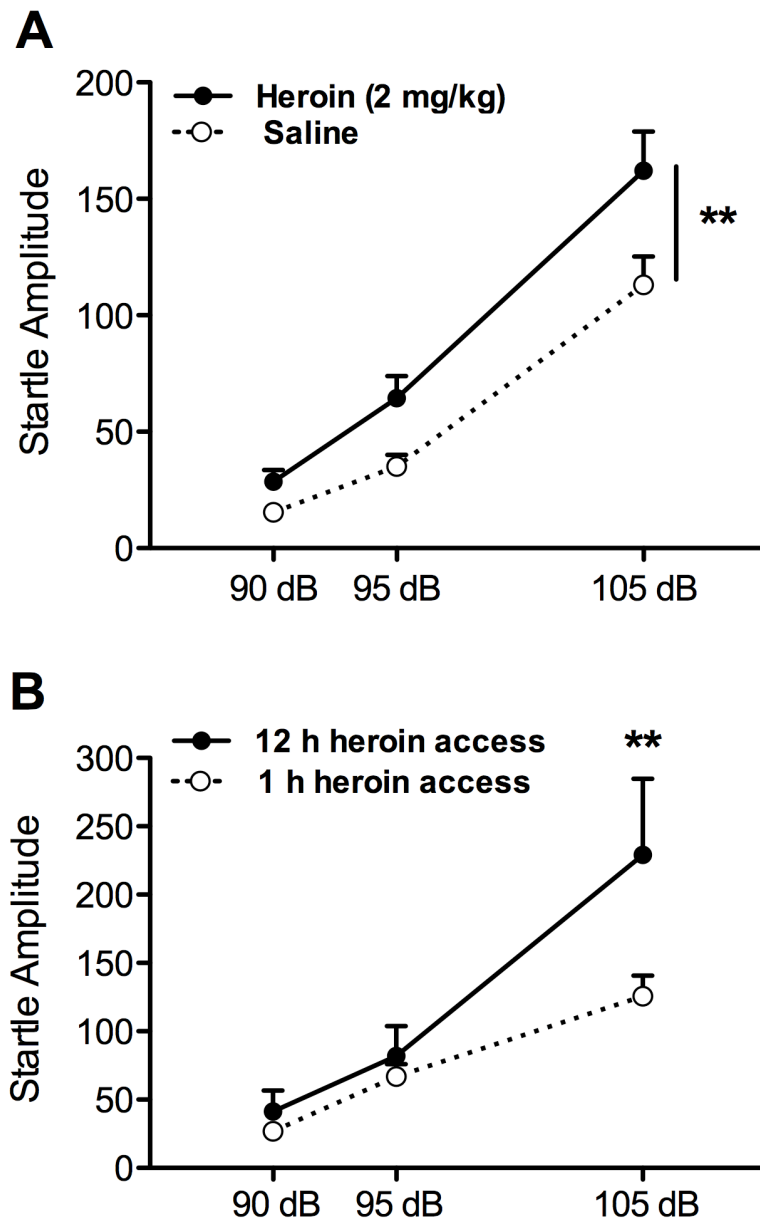
Figure 3-5 shows the effect of clonidine (10  $\mu$ g/kg, SC) on CRF-potentiated startle. The two-way repeated-measures ANOVA revealed a significant group  $\times$  treatment  $\times$  intensity interaction ( $F_{2,68} = 3.23, p < 0.05$ ). The *post hoc* analysis indicated that CRF significantly potentiated the ASR at 105 dB, and saline + ICV CRF produced significantly higher startle amplitudes compared with clonidine + ICV CRF ( $p < 0.0001$ ), saline + ICV saline ( $p < 0.0001$ ), and clonidine + ICV saline ( $p < 0.0001$ ). These results indicate that clonidine reversed CRF-potentiated startle, with no effect on its own. An effect of intensity was found

( $F_{2,68} = 76.53, p < 0.0001$ ), showing startle amplitudes at 90 dB < 95 dB < 105 dB.

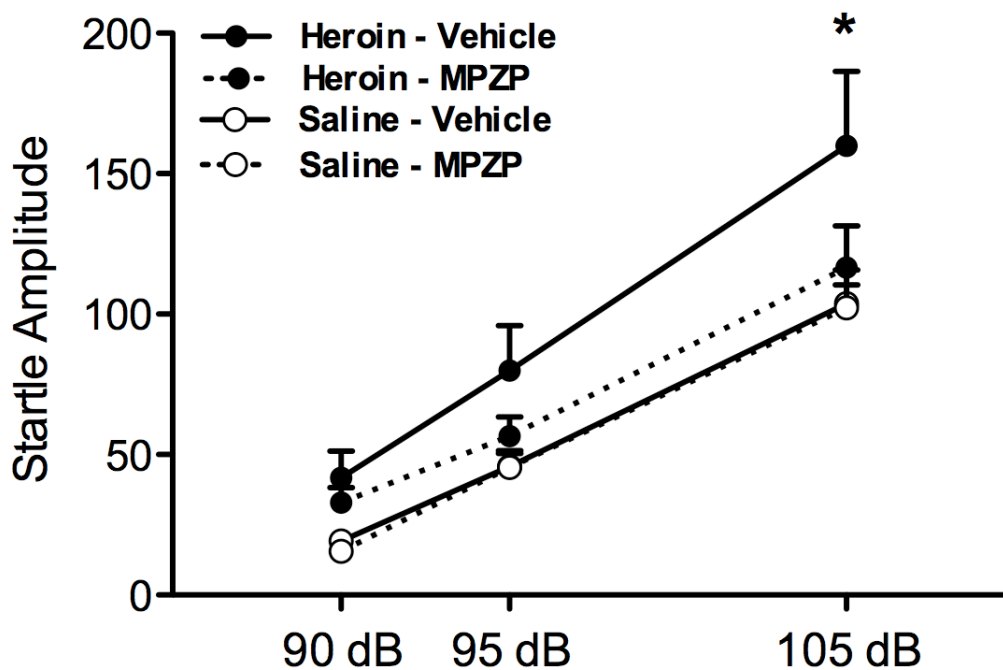


**Table 3-1.** Dose-response curve for yohimbine-potentiated startle. \* $p < 0.05$ , different from 0 mg/kg. + $p < 0.05$ , different from 0.3125 mg/kg; & $p < 0.05$ , different from 0.625 mg/kg.  $n = 8$ .

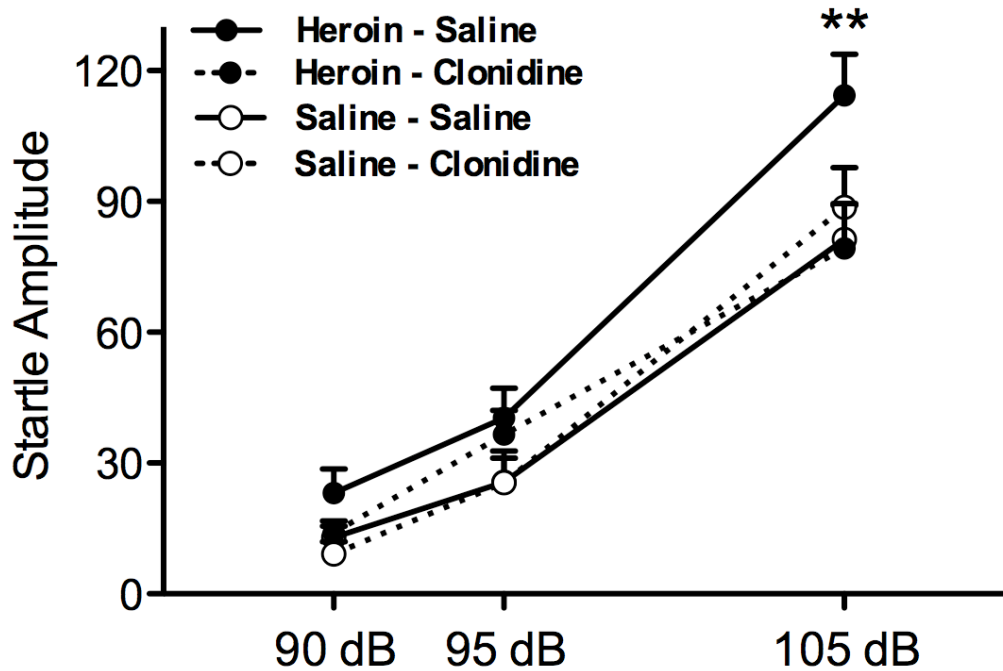
Yohimbine (mg/kg)	90 dB	95 dB	105 dB
0	23.1 ± 3.8	61.1 ± 10.5	131.6 ± 22.6
0.3125	24.5 ± 4.0	54.7 ± 8.6	144.6 ± 20.8
0.625	42.7 ± 7.8	101.7 ± 20.0*+	223.6 ± 39.9*+
1.25	56.1 ± 8.4*+	162.7 ± 24.6*+&	291.9 ± 39.3*+&



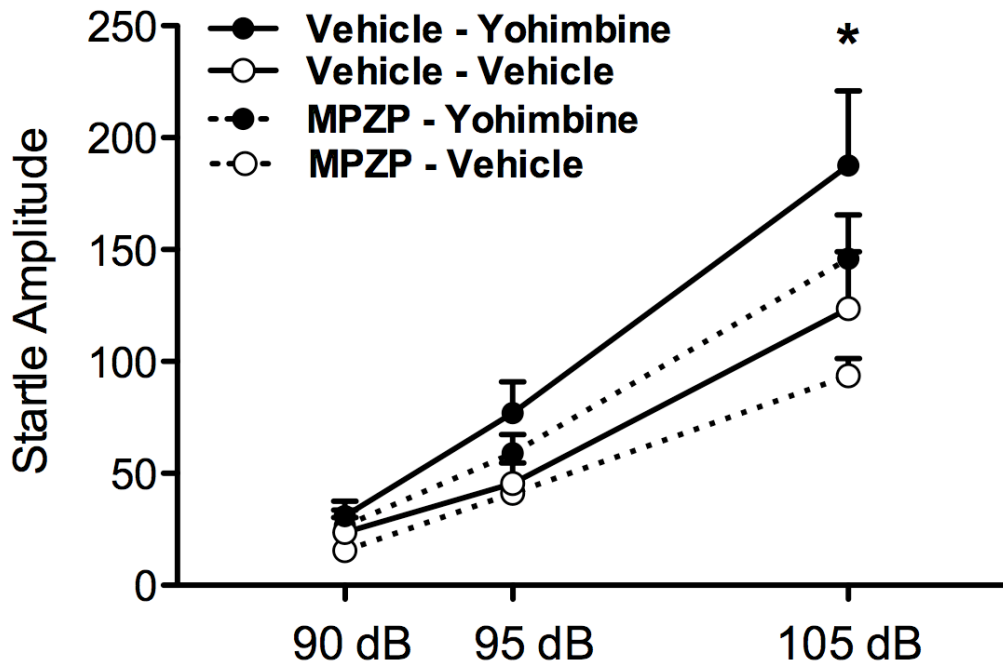
**Figure 3-1.** Heroin withdrawal-potentiated startle. (a) The ASR measured during withdrawal from a single injection of heroin (2 mg/kg, SC). The ASR was measured 4 h after the heroin injection. A significant difference in startle amplitude was found between saline- and heroin-treated rats across all intensities.  $n = 15$ .  $**p < 0.01$ , different from saline. (b) The ASR was measured 10-20 h into withdrawal from intravenous heroin self-administration. Withdrawal-potentiated startle was observed in rats with extended access (12 h) to heroin compared with rats with limited access (1 h) to heroin self-administration.  $n = 7-11$ .  $**p < 0.005$ , different from 1 h self-administration.



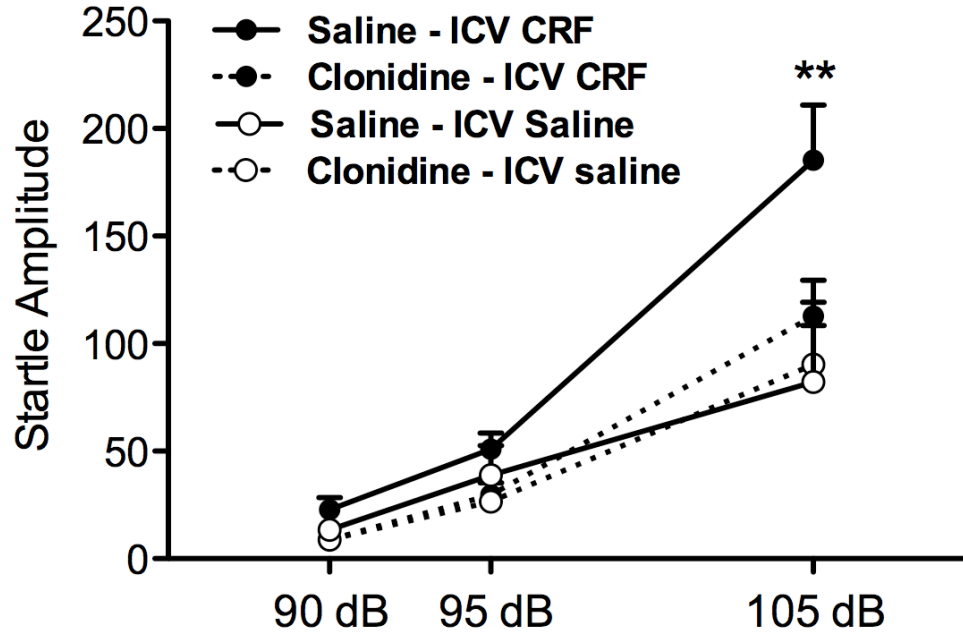
**Figure 3-2.** MPZP blocked heroin withdrawal-potentiated startle. The CRF<sub>1</sub> receptor antagonist MPZP (20 mg/kg, SC) blocked the heightened startle during withdrawal from heroin. The rats that received heroin-vehicle had significantly higher startle amplitudes compared with the heroin-MPZP, saline-vehicle, and saline-MPZP groups.  $n = 11-14$ .  $*p < 0.01$ , different from all groups.



**Figure 3-3.** Clonidine blocked heroin withdrawal-potentiated startle. Heroin-saline treatment produced a significantly higher ASR at 105 dB compared with rats that received heroin-clonidine, saline-saline, and saline-clonidine.  $n = 7-8$ . \*\* $p < 0.0005$ , different from all other groups.



**Figure 3-4.** MPZP did not block yohimbine-potentiated startle. Yohimbine (1.25 mg/kg, SC) significantly increased the ASR compared with vehicle-vehicle, MPZP-yohimbine, and MPZP-vehicle.  $n = 11$ . \* $p < 0.05$ , different from vehicle-vehicle.



**Figure 3-5.** Clonidine blocked CRF-potentiated startle. Rats that received ICV CRF exhibited a significant increase in the ASR compared with rats treated with saline-ICV saline, clonidine-ICV saline, and clonidine-ICV CRF.  $n = 7-12$ . \*\* $p < 0.005$ , different from clonidine-ICV saline group.

## DISCUSSION

We report that the ASR is increased during acute withdrawal from a single injection of heroin. Similarly, rats that underwent withdrawal from 1 day of extended access to intravenous heroin self-administration exhibited heightened startle compared with rats that had limited access to heroin self-administration. We found that both MPZP and clonidine were able to block withdrawal-potentiated startle produced by heroin, indicating a functional role for CRF<sub>1</sub> and  $\alpha$ -adrenergic receptors in mediating this effect. Additionally, we found that clonidine was able to block CRF-potentiated startle, whereas MPZP did not completely block yohimbine-potentiated startle, suggesting that CRF facilitates NE release to potentiate startle.

Previous studies observed a heightened startle response in rodents during withdrawal from ethanol (Rassnick *et al*, 1992), nicotine (Helton *et al*, 1993), diazepam (Rasmussen *et al*, 1994), and morphine (Harris and Gewirtz, 2004). Consistent with these findings, we found that the startle response increased during both acute withdrawal from a single subcutaneous injection of heroin and during withdrawal from extended access (12 h) to heroin self-administration. Rats with limited access (1 h) to heroin did not show an increased ASR. The rats with limited access self-administered approximately 0.3 mg/kg heroin in a 1 h session, whereas rats with extended access self-administered an average of 2 mg/kg heroin in a 12 h session. Interestingly, the same dose (2 mg/kg) of heroin injected in the acute model produced a similar increase in the startle response. The extended access model of heroin self-administration captures several

aspects of opioid addiction (Vendruscolo *et al*, 2011). Thus, the similar results obtained in the startle response during withdrawal from acute heroin injection and extended heroin self-administration support the premise that the acute heroin withdrawal model is a simple and reliable measure to investigate the pharmacological mechanisms that underlie potentiated startle during opioid withdrawal.

We hypothesized that CRF may play a role in the anxiogenic-like effect of heroin withdrawal. Previous studies indicated that the startle response is elevated by ICV CRF administration, and anxiolytics, such as chlordiazepoxide, block this effect (Swerdlow *et al*, 1986). CRF<sub>1</sub> receptor antagonists also blocked anxiety-like behavior measured in the elevated plus maze after ICV CRF administration (Zorrilla *et al*, 2002). In the self-administration model, CRF<sub>1</sub> antagonists reduced the escalation of self-administration of cocaine (Specio *et al*, 2008), ethanol (Funk *et al*, 2007), and heroin (Greenwell *et al*, 2009), as well as the hyperalgesia observed in ethanol- and heroin-dependent animals (Edwards *et al.*, 2012). CRF<sub>1</sub> receptor antagonists have been shown to reduce anxiety-like behavior in rats with high basal anxiety but not in rats with low basal anxiety-like behavior (Keck *et al*, 2001). These studies suggest that extrahypothalamic CRF plays an important role in anxiety-like behavior that is prominent during drug withdrawal. We found that MPZP, a brain-penetrant CRF<sub>1</sub> receptor antagonist, blocked withdrawal-potentiated startle, indicating that CRF plays a functional role in anxiety-like behavior during heroin withdrawal.

Given the interaction between CRF and NE systems, we hypothesized



that NE also plays a role in anxiety-like behavior during heroin withdrawal. Clonidine has been shown to produce a rapid and prolonged reduction of opiate withdrawal symptoms in humans (Gossop, 1988). In animals, clonidine has been shown to attenuate conditioned place aversion to opiate withdrawal (Schulteis *et al*, 1998) and block morphine withdrawal-potentiated startle (Harris and Gewirtz, 2004). Consistent with these results, we found that clonidine blocked heroin withdrawal-potentiated startle. Clonidine is an agonist at presynaptic  $\alpha_2$  receptors and decreases both NE release and sympathetic outflow. Our data suggest that CRF and NE modulate the potentiation of startle during heroin withdrawal, with the involvement of CRF<sub>1</sub> and  $\alpha_2$  receptors.

To further explore the mechanistic relationship between CRF and NE in potentiated startle responses, we tested the effects of MPZP on yohimbine-potentiated startle and clonidine on CRF-potentiated startle. We hypothesized that CRF is the driving force in NE release, leading to an enhanced ASR. Consistent with this hypothesis, the results showed that clonidine was able to block CRF-potentiated startle, whereas systemic MPZP administration only partially attenuated yohimbine-potentiated startle. Neither clonidine nor MPZP significantly altered ASR on its own. MPZP produced a slight decrease of ASR that was not statistically significant. The MPZP dose (20 mg/kg) used in the present study has been shown to effectively decrease alcohol intake, cocaine self-administration, and anxiety-like behavior in dependent rats (Specio *et al.*, 2008; Richardson *et al.*, 2008), and reduce heroin withdrawal-induced mechanical hypersensitivity (Edwards *et al*, 2012). Although these findings

strongly suggest that 20 mg/kg MPZP produces sufficient receptor occupancy to produce pharmacological effects, we cannot completely rule out a possible interaction of CRF<sub>1</sub> receptors on yohimbine-potentiated startle. These results provide evidence that the CRF and NE systems interact to modulate anxiogenic-like effects expressed through the startle response and that CRF potentially drives NE release in the brain stress system to produce this behavioral effect. These results fit with the feed-forward CRF-NE-CRF stress system linking the forebrain (specifically the CeA, BNST, and paraventricular nucleus of the hypothalamus) and brainstem (locus coeruleus), whereby CRF can activate brainstem NE that in turn activates forebrain CRF (Koob, 1999). Evidence suggests that the balance between CRF and opioids may be important in limiting the noradrenergic response to stressors, and disruptions in this balance, such as chronic opioid use or stress, alters the balance in favor of CRF activation (Valentino and Van Bockstaele, 2001). Therefore, during opioid withdrawal, CRF may activate the CRF-NE feed-forward brain stress system, leading to a heightened noradrenergic response and increased ASR.

The neurocircuitry involved in the CRF-NE stress system modulation of heroin withdrawal-potentiated startle is a subject for future investigations. Our hypothesis is that the extended amygdala, specifically the CeA and BNST, comprises a key area that mediates anxiety-like behavior during withdrawal. CRF and NE are abundant in the CeA/BNST, and acute withdrawal from all major drugs of abuse increases CRF/NE release in these regions (Koob, 2008). CRF injected directly into the BNST enhanced the startle response, and lesions of the

BNST blocked CRF-potentiated startle (Lee and Davis, 1997). Additionally, infusion of the CRF antagonist  $\alpha$ -helical CRF<sub>9-41</sub> into the BNST blocked CRF-potentiated startle (Lee and Davis, 1997). These results suggest that the BNST may be a primary receptor site for the excitatory effects of ICV CRF on the ASR. Given these findings, it would be interesting to determine whether manipulations of the CRF and NE systems in the BNST and CeA alter heroin withdrawal-potentiated startle. Furthermore, ASR has been shown to be potentiated by naloxone for at least 80 days into withdrawal (protracted abstinence) after a single morphine exposure (Rothwell *et al.*, 2012), thus, investigating the effects of MPZP and clonidine during protracted withdrawal would be an interesting future study.

In conclusion, we demonstrated that the acoustic startle response is potentiated during withdrawal from a single heroin administration and from extended access to heroin self-administration. Both clonidine, by decreasing NE release, and MPZP, by antagonizing CRF<sub>1</sub> receptors, blocked heroin withdrawal-potentiated startle. Altogether, these results suggest that CRF may drive the activation of the feed-forward CRF-NE-CRF brain stress system to produce anxiety-like behavior during opioid withdrawal. Pharmacological manipulation of the CRF and NE systems may be useful in the alleviation of anxiety during withdrawal and may ultimately help in the treatment of opioid addiction.

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## **CHAPTER 4**

**Development of mechanical hypersensitivity in rats during heroin and ethanol dependence: alleviation by CRF<sub>1</sub> receptor antagonism**



**ABSTRACT**

Animal models of drug dependence have described both reductions in brain reward processes and potentiation of stress-like (or anti-reward) mechanisms, including a recruitment of corticotropin-releasing factor (CRF) signaling. Accordingly, chronic exposure to opiates often leads to the development of mechanical hypersensitivity. We measured paw withdrawal thresholds (PWTs) in male Wistar rats allowed limited (short access group: ShA) or extended (long access group: LgA) access to heroin or cocaine self-administration, or in rats made dependent on ethanol via ethanol vapor exposure (ethanol-dependent group). In heroin self-administering animals, after transition to LgA conditions, thresholds were reduced to around 50% of levels observed at baseline, and were also significantly lower than thresholds measured in animals remaining on the ShA schedule. In contrast, thresholds in animals self-administering cocaine under either ShA (1 h) or LgA (6 h) conditions were unaltered. Similar to heroin LgA rats, ethanol-dependent rats also developed mechanical hypersensitivity after eight weeks of ethanol vapor exposure compared to non-dependent animals. Systemic administration of the CRF1R antagonist MPZP significantly alleviated the hypersensitivity observed in rats dependent on heroin or ethanol. The emergence of mechanical hypersensitivity with heroin and ethanol dependence may thus represent one critical drug-associated negative emotional state driving dependence on these substances. These results also suggest a recruitment of CRF-regulated nociceptive pathways associated with escalation of intake and dependence. A greater understanding of

relationships between chronic drug exposure and pain-related states may provide insight into mechanisms underlying the transition to drug addiction, as well as reveal new treatment opportunities.

## **INTRODUCTION**

### *Drug Addiction and Negative Motivational Symptomatology*

Drug addiction (or substance dependence, DSM-IV) is a chronic relapsing disorder characterized by a persistent compulsion to seek and take drugs (Self and Nestler, 1995) and the recruitment of negative motivational (or anti-reward) mechanisms that manifest during abstinence (Koob and Le Moal, 1997).

Addiction can be conceptualized as a progressive disorder whereby drugs of abuse are initially taken for their pleasurable effects, although tolerance to these effects will often develop over time. The parallel development of intense negative emotional states (e.g., anxiety, dysphoria, irritability) as the addiction timeline progresses may represent the final definitive stage of this disease. Such conditions may further drive excessive drug-seeking behavior even as tolerance to the positive rewarding effects of drugs persists as a consequence of within- and between-circuit neuroadaptations (Edwards and Koob, 2010).

### *Pain as a Negative Motivational Symptom Influencing Drug Addiction*

Chronic pain affects approximately one third of the U.S. adult population (Johannes et al., 2010), a number that will likely increase over the next several decades given an aging population in several western countries including the

United States. Effective pain management is a central aim of medical care, with opioids remaining the best resource for analgesia and overall improvement of quality of life in chronic pain patients. Unfortunately, chronic opiate exposure intended to alleviate pain often paradoxically renders individuals more sensitive to nociceptive stimuli, a condition termed opioid-induced hyperalgesia (extensively reviewed in Angst and Clark, 2006). Importantly, hyperalgesia is also prevalent in former opiate addicts maintained on methadone, further suggesting that this condition emerges with protracted opiate use itself. In accordance with this hypothesis, heroin exposure in rodents leads to a spontaneous reduction in mechanical nociceptive thresholds (Laulin et al., 1998) that is exacerbated after chronic treatment (Simonnet and Rivat, 2003), suggesting a recruitment or sensitization of pro-nociceptive systems (Celerier et al., 2001). Given that chronic pain is well known to cause both emotional distress and produce a sustained negative emotional state (King et al., 2009), opiate-induced hyperalgesia may constitute a condition intimately associated with the transition to drug dependence by facilitating negative reinforcement processes.

The rise in prescription analgesic abuse and dependence (Maxwell, 2011) further suggests a foreboding link between pain modulation and addiction. Indeed, a long-standing concern for medical practitioners is how to administer chronic opioid analgesics to pain patients without generating an unwanted dependence or addiction (Fields, 2011). Fortunately, opioid pharmacotherapy rarely leads to abuse following discontinuation of therapy; however, the risk of drug addiction is enhanced in patients with a history of illicit opioid use or abuse

(Fishbain et al., 2008; Turk et al., 2008). Thus, hyperalgesia may exacerbate or maintain addicted states (drug dependence and compulsive drug-seeking behaviors) in this more compromised population. Shurman, Koob, and Gutstein (2010) further hypothesize that effective pain management with doses of opioids that are strictly titrated to the relief of pain represents the best treatment strategy, whereas any over-exposure to opioids in excessive amounts (i.e., above physiological needs) may lead to the recruitment of opponent motivational processes in terms of both pain (hyperalgesia) and negative affect (termed hyperkatifeia). Such opponent neuroadaptations are apparent at even the cellular level following opiate exposure (Bederson et al., 1990; Kaplan and Fields, 1991; Nestler and Aghajanian, 1997), but may also progress to the recruitment of multiple brain stress circuits during dependence (Koob, 2008). Targeting neuroadaptations at either or both of these levels may represent viable anti-dependence therapeutic strategies in the future (McClung, 2006; Koob et al. 2009; Heilig et al., 2010).

Similar to dependence on opiates, alcoholism (or ethanol dependence) is a chronically relapsing mental disorder involving profound motivational disturbances and a loss of control over drinking. Alcoholism is also often accompanied by the emergence of negative emotional states that constitute a motivational withdrawal syndrome when access to alcohol is disrupted (Gilpin and Koob, 2008). The limited, recreational use of alcohol seen in a majority of users is clinically distinct from the escalated drinking, loss of control, and emergence of compulsive ethanol-seeking behaviors that characterize

alcoholism. Chronic alcohol use impacts several peripheral and central nervous system actions, and while it has long been observed that oral alcohol administration increases human pain thresholds (Wolff et al., 1942), withdrawal from chronic use often results in increased pain sensitivity as one component of a larger alcohol withdrawal syndrome (Jochum et al., 2010). In fact, polyneuropathy, characterized by axonal degeneration and demyelination, is the most common neurological complication in alcoholics (Diamond and Messing, 1994). Such data suggest that drinking in alcoholics may in part be motivated by a desire to alleviate ethanol withdrawal-induced hyperalgesia. Indeed, self-reports of alcohol use specifically for pain management are common, even in young adults (e.g., Riley and King, 2009). In one study (Brennan et al., 2005), problem drinkers not only described more severe pain symptoms compared to non-drinkers, they also reported a higher incidence of using alcohol to manage their pain. Moreover, follow-up studies indicated that the use of alcohol to manage pain resulted in a worsening of drinking and other health-related problems three years later, indicating that negative reinforcement mechanisms related to pain-targeted drinking could underlie the persistence and exacerbation of both alcohol-related disorders and overall life morbidity.

#### *Animal Models of Drug Dependence: Recruitment of CRF<sub>1</sub>R Signaling*

A critical breakthrough in the ability to examine negative reinforcement theories of drug addiction has been the development and refinement of reliable animal models of excessive drug (Ahmed and Koob, 1998) and alcohol (Gilpin et

al., 2008) self-administration. For example, prolonged access to heroin self-administration (6-23 h per day) leads to escalation of heroin intake and dependence in rats, while animals given restricted access (1 h per day) fail to escalate intake (Edwards et al., 2009; Vendruscolo et al., 2011). Escalated animals go on to display a prolonged latency to extinguish heroin self-administration (a measure of drug seeking) as well as enhanced sensitivity to stress- and heroin-induced reinstatement (Ahmed et al., 2000; Lenoir and Ahmed, 2007). Similarly, in one model of ethanol dependence, rats trained to self-administer ethanol exhibit substantial increases in operant self-administration following chronic intermittent ethanol vapor exposure (Rogers et al., 1979; Gilpin et al., 2008). Ethanol vapor exposure allows for the reliable titration of blood alcohol levels (BALs) that are sufficient for inducing ethanol dependence, as indicated by both somatic withdrawal signs (Roberts et al., 2000) and negative motivational symptoms (Schulteis et al., 1995) during acute withdrawal. As a cardinal mediator of the magnified stress response that manifests during drug withdrawal, the recruitment of corticotropin-releasing factor (CRF), a neuropeptide critical to stress signaling, represents a central pharmacological system contributing to the establishment and maintenance of both heroin and alcohol dependence (Logrip et al., 2011). Chronic administration of opiates or ethanol leads to withdrawal-induced increases in brain CRF levels (Weiss et al., 2001; Funk et al., 2006; Merlo-Pich et al., 1995; Olive et al., 2002). Moreover, pharmacological blockade of CRF<sub>1</sub> receptors reduces both ethanol self-administration in ethanol-dependent animals (Funk et al., 2007; Richardson et

al., 2008) and escalated heroin self-administration in heroin-dependent rats (Greenwell et al., 2009). CRF signaling mediates the expression of various withdrawal-related behaviors associated with both heroin (Heinrichs et al., 1995; Stinus et al., 2005) and alcohol (Valdez et al., 2002; Valdez et al., 2003; Huang et al., 2010) dependence, although the regulation of drug withdrawal-induced modulation of pain-related processes by CRF<sub>1</sub>R signaling has never been examined.

### *Development of Mechanical Hypersensitivity During the Transition from Drug Use to Dependence*

The current study tracked changes in paw withdrawal thresholds (PWTs) across an extended timeline designed to model the natural development of, or progression to, drug dependence. Tests were conducted before and after exposure to three common drugs of abuse, and more importantly, before and after transition to extended access conditions modeling the escalating intake patterns associated with drug addiction. Finally, given the role of CRF signaling in the establishment and maintenance of dependence-related symptomatology, the ability of CRF<sub>1</sub>R antagonism to modify altered mechanosensitivity in dependent vs. non-dependent animals was also determined.

## **MATERIALS AND METHODS**

### *Animals*

Male Wistar rats (n = 98, Charles River) initially weighting 275-325g were communally housed (2-3/cage) with food and water available *ad libitum*. The animals were housed in a temperature-controlled (21.5°C) vivarium and maintained on a 12 h light/dark cycle (lights on at 0800). Animals were regularly handled for one week before surgery or the onset of operant training. All experiments adhered to the guidelines provided in the NIH *Guide for the Care and Use of Laboratory Animals* and protocols were reviewed and approved by The Scripps Research Institute's Institutional Care and Use Committee.

#### *Ethanol Self-Administration and Ethanol Vapor Chamber Procedures*

Animals were first trained to self-administer 10% (w/v) ethanol and water solutions until a stable response was maintained. To facilitate acquisition of operant self-administration, rats were initially provided 2-bottle choice access to ethanol (10% w/v) and water for 48 hours in the home cage. Rats then received 1 h 2-bottle choice access, time-matched to the future operant sessions, for 4 days. Rats acquired operant self-administration behavior during a single 12-h session, in which responses on the active lever yielded delivery of 0.1 ml water while responses on the inactive lever generated no scheduled consequences. The training session spanned one dark cycle with chow available *ad libitum* and yielded rapid acquisition of operant self-administration behavior. Following stable acquisition of operant ethanol self-administration (twelve sessions), animals were then split into two groups that were matched for ethanol self-administration over their last three sessions, with one group designated as “ethanol-dependent”



(vapor-exposed, n = 18) and the other as “non-dependent” (air-exposed, n = 12). Ethanol vapor exposure has been demonstrated to be a safe, reliable method to titrate blood alcohol levels (BALs) for the induction of ethanol dependence and has significant construct validity (Gilpin et al., 2008). In this procedure, ethanol exposure adjusted by the experimenter to maintain dependence-inducing BALs without jeopardizing animal health, and BALs were regulated between 150-250 mg%. Tail blood samples were taken and analyzed 1-2x per week for BAL determination as previously described (Gilpin et al., 2008). Animals underwent cycles of 14 h on vapor and 10 h off. During withdrawal, vapor-exposed animals exhibit a host of symptoms related to dependence, including both somatic withdrawal signs (Roberts et al., 2000) and negative motivational symptoms (Schulteis et al., 1995), and show excessive alcohol drinking compared to air-exposed animals at a time when their BALs approach zero (Edwards et al., 2011).

#### *Intravenous Drug Self-Administration Procedures*

After acclimation to the housing environment, rats were implanted with a silastic catheter into the right external jugular vein as described (Orio et al., 2009) and allowed to recover 1 week before baseline paw withdrawal testing. Food and water were available *ad libitum* throughout the study. Self-administration sessions were conducted in an operant conditioning chamber, which was placed in a light- and sound-attenuating cubicle (28 × 26 × 20 cm; Med Associates Inc., St Albans, VT). Rats were allowed to self-administer either cocaine (0.5

mg/kg/infusion, n = 18) or heroin (0.06 mg/kg/infusion, n = 44) in 13 daily, 1-h training sessions over two weeks on a fixed-ratio 1 (FR1) schedule. Following stable acquisition of self-administration, animals were divided into ShA (1 h self-administration for both cocaine and heroin) and LgA (6 h self-administration for cocaine and 12 h self-administration for heroin) groups for subsequent sessions. Twelve hours of access to heroin was used based on previous studies showing that this time is optimal for producing escalation of heroin intake (Vendruscolo et al., 2011).

#### Measurement of Mechanosensitivity (Paw Withdrawal Thresholds)

Mechanosensitivity testing was conducted before drug exposure (baseline, BL) and during withdrawal, just prior to subsequent self-administration sessions. Evaluation of paw withdrawal thresholds (PWTs) was performed according to methods described by Chaplan et al. (1994). Briefly, rats were acclimated for 15 min in elevated cages with a wire mesh floor. A series of von Frey filaments were applied perpendicularly to the plantar surface of the hind paw for 3 seconds. A sharp withdrawal of the hind paw indicated a positive response. The stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed in order to determine a pattern of responses to apply to the statistical method of Dixon (1980). The 50% paw withdrawal threshold was determined by the formula  $X_f + k\delta$ , where  $X_f$  = last von Frey filament employed,  $k$  = Dixon value corresponding to response pattern, and  $\delta$  = mean difference between stimuli.

Baseline mechanical nociceptive thresholds were similar to those reported for the ages of rats employed in this study (Ririe and Eisenach, 2006).

### Pharmacological Testing of Altered Mechanosensitivity

The CRF<sub>1</sub>R antagonist N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo[1,5-a]pyrimidin-7-amine (MPZP) was prepared for systemic administration by first solubilizing it in 1 M HCl (10% final volume). It then was diluted using 25% w/v hydroxypropyl  $\beta$ -cyclodextrin (HBC, Cargill, Cedar Rapids, IA) (80% final volume) and backtitrated with descending concentrations of NaOH (2, 1, 0.1 M) (10% final volume) resulting in a final suspension of 10 mg/ml MPZP in 20% HBC (pH 4.5). Lower concentrations were then prepared by serial dilution with vehicle (20% HBC, pH 4.5). Animals were administered the appropriate dose via a 2 ml/kg injection (0–20 mg MPZP/2 ml vehicle/kg body weight, subcutaneously) in a Latin-square, dose-counterbalanced design 60 minutes before testing. For the 0 mg/kg dose of MPZP, animals were given 2 ml 20% HBC vehicle/kg body weight. Doses and pretreatment times were based on previous studies of CRF<sub>1</sub>R antagonist effects on reducing excessive ethanol drinking (Richardson et al., 2008) and antinociception (Ji et al., 2007).

### Statistics

Escalation of cocaine or heroin self-administration was compared across sessions using repeated-measures one-way ANOVA followed by Dunnett's

multiple comparison tests. Differences in PWTs between LgA and ShA animals or between ethanol-dependent and non-dependent animals were compared using repeated-measures two-way ANOVA (group x test day or group x MPZP dose) followed by a Bonferroni post hoc test. Differences in PWTs in LgA or ethanol-dependent animals across tests were analyzed using repeated-measures one-way ANOVA followed by Student-Newman-Keuls multiple comparison tests. Direct comparisons between individual preferred levels of heroin intake during the last session and subsequent individual PWTs were analyzed by Pearson correlation. All statistical analyses were performed with Prism 4.0c.

### Experimental Design

Figure 1 shows a schematic timeline for the experiments conducted in this study. For intravenous drug self-administration studies, paw withdrawal threshold (PWT) tests (indicated by arrows) were conducted before (baseline, BL) and after the initiation of drug exposure. Tests 1 & 2 were conducted during the training phase (1h, ShA conditions for all animals) following sessions six and thirteen, respectively. Animals were divided into ShA and LgA groups at this point (balanced according to drug self-administration levels and extant PWTs), and subsequent mechanosensitivity tests (3 & 4 after post-transition sessions seven and fifteen, respectively) were conducted during this LgA escalation phase. In a separate experiment, rats (n=17) were allowed to self-administer heroin under the same schedule, and the effect of CRF<sub>1</sub>R antagonism on PWTs

was subsequently determined. For ethanol studies, rats were first trained to self-administer alcohol as described in the methods section. Mechanosensitivity tests were conducted at baseline (before ethanol self-administration) and then before and after chronic intermittent ethanol vapor exposure (ethanol-dependent group) or air exposure (non-dependent group). Following ten weeks of vapor exposure, the effect of CRF<sub>1</sub>R antagonism on PWTs was subsequently tested in ethanol-dependent and non-dependent animals during weeks eleven and twelve of vapor exposure.

## RESULTS

### Paw Withdrawal Thresholds in Heroin Self-Administering Animals

Animals trained to self-administer heroin (0.06 mg/kg/infusion) under ShA (1 h) conditions displayed a limited intake (Figure 4-2A) yet developed a modest reduction in PWTs that was not significantly different from baseline levels (Figure 4-2B), although it appeared to persist for the duration of the experiment. Animals that transferred to extended access (LgA, 12 h) heroin self-administration displayed an escalation of intake (one-way repeated-measures ANOVA significant effect of session in LgA group,  $F_{14, 224} = 10.66$ , Figure 2A) that became significant by session eight ( $p < 0.01-0.05$ , Dunnett's multiple comparison tests). Concomitantly, PWTs in LgA animals were also significantly lower compared to animals remaining on the ShA (1 h) schedule (two-way repeated-measures ANOVA significant main effect of group,  $F_{1, 48} = 4.535$ , Figure 2B). Heroin LgA

animals developed a significant mechanical hypersensitivity by PWT test 3 (i.e., after seven self-administration sessions), and PWTs continued to decline after eight more sessions of LgA heroin self-administration at PWT test 4 (one-way repeated-measures ANOVA significant effect of test day in LgA group,  $F_{14, 44} = 4.026$ ; significantly lower PWTs at PWT 3 vs. BL,  $q = 2.903$ ,  $p < 0.05$ ; significantly lower PWTs at PWT 4 vs. BL,  $q = 3.851$ ,  $p < 0.05$ ; Figure 2B). Overall, these changes in mechanical hypersensitivity appeared to mirror the escalation of heroin intake in LgA rats. In fact, after 15 sessions on the LgA schedule, individual PWTs in heroin-dependent rats at PWT 4 were significantly and negatively correlated with preferred levels of heroin self-administration during the last session (Figure 4-2C,  $r = -0.609$ ,  $p = 0.016$ ). Given the role of CRF receptor signaling in the escalation of heroin intake (Greenwell et al., 2009) and heroin withdrawal-associated behaviors (Heinrichs et al., 1995; Stinus, et al., 2005) including thermal hyperalgesia in precipitated morphine withdrawal (McNally and Akil, 2002), we next tested the effects of systemic CRF<sub>1</sub>R antagonism on altered mechanosensitivity during early (10-12 h) spontaneous withdrawal in LgA animals. In a separate group of rats ( $n=17$ ), previously lowered PWTs in LgA rats (two-way repeated-measures ANOVA significant main effect of group,  $F_{1, 30} = 11.14$ ) were significantly elevated near levels observed in ShA rats after systemic administration of MPZP (Figure 4-2D). One-way ANOVA conducted on LgA rat thresholds revealed a significant improvement in mechanical hypersensitivity after administration of the 20 mg/kg MPZP dose that constituted 61.9% of the difference in thresholds between LgA and ShA groups

following vehicle treatment (one-way repeated-measures ANOVA significant effect of dose,  $F_{2, 26} = 4.186$ ; significantly higher PWTs after administration of 20 mg/kg MPZP vs. 0 mg/kg,  $q = 3.932$ ,  $p < 0.05$ ).

#### *Paw Withdrawal Thresholds in Cocaine Self-Administering Animals*

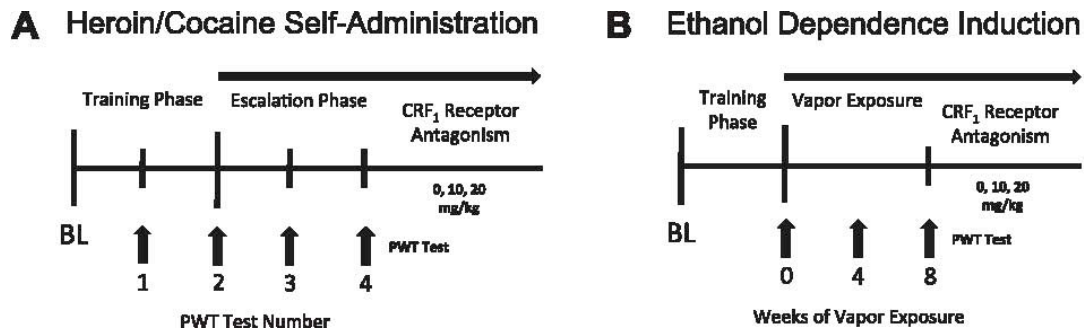
LgA cocaine (6 h) self-administering animals displayed a significant intake escalation (one-way repeated-measures ANOVA significant effect of session in LgA group,  $F_{14, 134} = 6.892$ , Figure 4-3A) that was apparent by session seven ( $p < 0.01$ , Dunnett's multiple comparison tests). However, in contrast to LgA heroin self-administering animals, LgA cocaine animals failed to develop altered mechanosensitivity over fifteen sessions of LgA conditions (Figure 4-3B).

#### *Paw Withdrawal Thresholds in Ethanol-Dependent and Non-Dependent Animals*

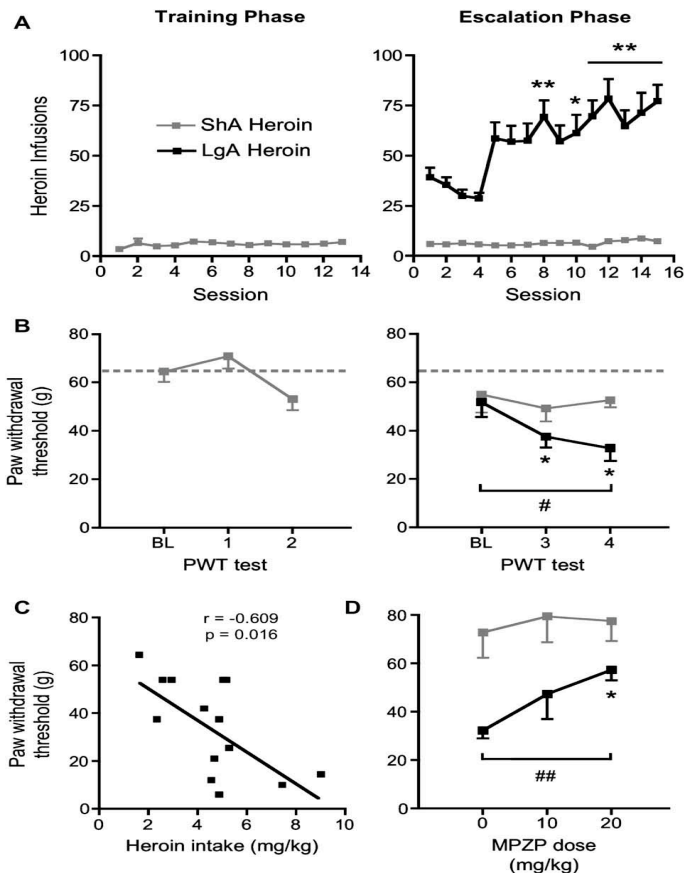
We next sought to determine the relationship between the induction and maintenance of ethanol dependence and mechanical hypersensitivity (Figure 4-4). Animals were initially trained to self-administer alcohol, and subsequently, half of the animals were made dependent via chronic, intermittent ethanol vapor exposure (Gilpin et al., 2008). The time course of weekly BALs in ethanol-dependent animals is presented in Figure 4-4A. While limited ethanol self-administration (average ethanol intake ranging from 0.54-0.66 g/kg on the last 3 of 12 training sessions) failed to alter PWTs (BL vs. 0-week vapor tests), animals subsequently made dependent on ethanol displayed reduced PWTs by eight

weeks of vapor exposure (two-way repeated measures ANOVA significant group x time interaction,  $F_{2, 68} = 3.266$ ; significant difference at 8-week PWT test by Bonferroni post hoc test,  $t = 2.722$ ; Figure 4-4B), similar to a previous study utilizing an ethanol liquid diet to induce dependence (Dina et al., 2000). Given the role of stress-related signaling in the development of ethanol withdrawal-related behaviors (Valdez et al., 2002; Valdez et al., 2003; Huang et al., 2010), including altered mechanosensitivity (Dina et al., 2008), we tested the effects of CRF<sub>1</sub>R antagonism on compromised thresholds in ethanol-dependent animals. During weeks 11-12 of ethanol vapor exposure, the lowered PWTs in ethanol-dependent rats (two-way repeated-measures ANOVA significant main effect of group,  $F_{1, 66} = 21.70$ ) were significantly elevated by systemic administration of the CRF<sub>1</sub>R antagonist MPZP (Figure 4-4C). One-way ANOVA conducted on ethanol-dependent rat thresholds revealed a significant improvement in mechanical hypersensitivity after administration of the 20 mg/kg MPZP dose that constituted 54.9% of the difference in thresholds between ethanol-dependent and non-dependent groups following vehicle treatment (one-way repeated-measures ANOVA significant effect of dose,  $F_{2, 35} = 3.733$ ; significantly higher PWTs after administration of 20 mg/kg MPZP vs. 0 mg/kg,  $q = 3.823$ ,  $p < 0.05$ ). The efficacy of MPZP in alleviating heroin and alcohol withdrawal-induced mechanosensitivity approximated the efficacy of another CRF<sub>1</sub>R antagonist (NBI 27914) in reducing hyperalgesia in an arthritis pain model (Ji et al., 2007).

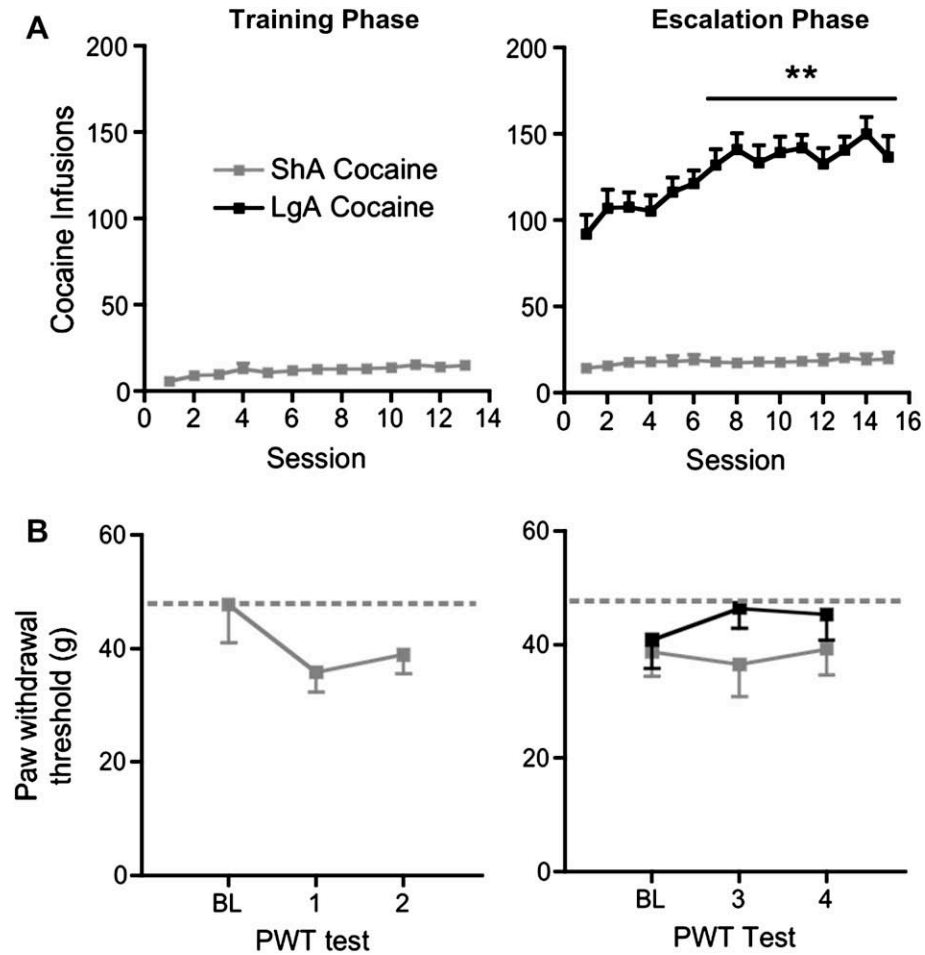




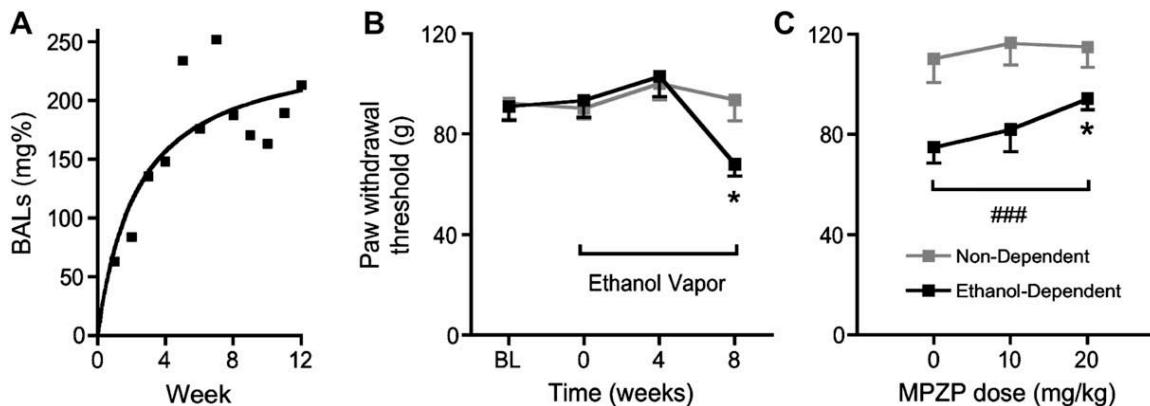
**Figure 4-1.** Experimental timelines. (A) For heroin and cocaine self-administration studies, repeated paw withdrawal threshold (PWT) tests (indicated by arrows) were conducted before (baseline, BL) and during 1 h ShA training sessions. Subsequently, animals were divided into ShA and LgA groups, and PWT tests were conducted over this escalation phase. In a separate group of heroin self-administering animals, the effect of the CRF<sub>1</sub>R antagonist MPZP on mechanosensitivity was determined post-escalation. (B) For the alcohol study, animals were tested before (baseline, BL) and after ethanol self-administration (0 weeks vapor exposure), and following 4 and 8 weeks of vapor exposure. Subsequently, the effect of CRF<sub>1</sub>R antagonism on mechanosensitivity was determined over weeks 11-12 of vapor exposure.



**Figure 4-2.** Heroin self-administration and paw withdrawal thresholds in ShA and LgA animals. (A) Animals acquired heroin self-administration ( $n = 27$ ) and upon transition to LgA conditions (12 h access,  $n = 15$ ) significantly increased heroin intake. Asterisks indicate  $**p < 0.01$  or  $*p < 0.05$  significantly higher intake in LgA animals versus session one. (B) Paw withdrawal threshold (PWT) tests conducted during withdrawal revealed a development of mechanical hypersensitivity selectively in LgA animals following 7 and 15 sessions of extended access conditions (PWT tests 3 & 4, respectively). Pound sign indicates  $\#p < 0.05$  significantly lower PWTs in LgA vs. ShA animals (main effect of group). Asterisk indicates  $*p < 0.05$  significantly lower PWTs in LgA animals versus baseline (BL) test. (C) Individual paw withdrawal thresholds at PWT 4 were significantly and negatively correlated with individual preferred levels of heroin intake during session 15 in LgA animals ( $r = -0.609$ ,  $p = 0.016$ ). (D) In another group of escalated LgA animals ( $n = 9$ ) that displayed a significant reduction in paw withdrawal thresholds vs. ShA animals ( $n = 8$ ), the CRF<sub>1</sub>R antagonist MPZP significantly raised PWTs near levels measured in the ShA group, corresponding to a putative alleviation of heroin withdrawal-induced mechanical hypersensitivity. Pound signs indicate  $\#\#p < 0.01$  significantly lower PWTs in LgA vs. ShA animals (main effect of group). Asterisks indicate  $*p < 0.05$  significant elevation of PWTs in LgA animals.



**Figure 4-3.** Cocaine self-administration and paw withdrawal thresholds in ShA and LgA animals. (A) Animals acquired cocaine self-administration ( $n = 18$ ) and upon transition to LgA conditions (6 h access,  $n = 9$ ) significantly increased cocaine intake. Asterisks indicate  $**p < 0.01$  significantly higher intake in LgA animals versus session one (B) Paw withdrawal threshold (PWT) tests conducted during withdrawal did not significantly change over the course of cocaine self-administration under either ShA or LgA conditions.



**Figure 4-4.** Ethanol vapor exposure and paw withdrawal thresholds in ethanol-dependent and non-dependent animals. (A) Blood alcohol levels (BALs) of ethanol-dependent animals ( $n = 18$ ) over the course of the experiment. (B) Paw withdrawal thresholds were not altered by ethanol self-administration (baseline test vs. 0-week vapor exposure test). Following eight weeks of ethanol vapor exposure, paw withdrawal thresholds in ethanol-dependent animals ( $n = 18$ ) were significantly reduced compared to non-dependent animals ( $n = 18$ ). Asterisk indicates  $*p < 0.05$  significantly lower thresholds in ethanol-dependent vs. non-dependent animals at the eight week test. (C) During weeks 11-12 of ethanol vapor exposure, CRF<sub>1</sub>R antagonism with MPZP significantly elevated the compromised paw withdrawal thresholds in ethanol-dependent animals ( $n = 12$ ) near levels measured in the non-dependent group ( $n = 12$ ), representing a partial alleviation of ethanol withdrawal-induced mechanical hypersensitivity. Pound signs indicate  $###p < 0.001$  significantly lower paw withdrawal thresholds in ethanol-dependent vs. non-dependent animals (main effect of group). Asterisk indicates  $*p < 0.05$  significant elevation of thresholds in ethanol-dependent animals.

## DISCUSSION

### *Enhanced Mechanosensitivity as a Negative Motivational Symptom of Dependence*

The elucidation of opiate- and alcohol-induced neuroadaptations within brain reward and stress systems has provided valuable insights into potential mechanisms underlying drug dependence in vulnerable individuals. In these circuits, reward neurotransmitter systems including certain opioid peptides are compromised, while brain stress systems such as CRF signaling are recruited (Koob and Le Moal, 2008). Strong evidence suggests that the neural substrates associated with addiction may also overlap with substrates of emotional aspects of nociceptive processing in areas such as the central amygdala (Neugebauer et al., 2004), cingulate cortex (Vogt, 2005), and nucleus accumbens (Gear and Levine, 1995; Barrot et al., 2002), where ascending pain pathways connect for processing the emotional components of pain perception. Specifically, pain-responsive neurons are abundant in the lateral part of the central amygdala (also known as the “nociceptive amygdala”; Bernard and Besson, 1990). As a potentially powerful negative motivational symptom, pain represents a subjective experience that can have a substantial influence on drug reinforcement, possibly facilitating the transition to drug addiction (Miller and Gold, 2007). Although mechanisms of nociception, stress, and anxiety are difficult to dissociate, pain represents a unique negative affective valence and is often intimately associated with a host of psychiatric disorders (Elman et al., 2011). Moreover, agents that

alleviate pain-like states can be rewarding by acting via negative reinforcement mechanisms in animal models (King et al., 2009).

Our results describe significant increases in mechanical hypersensitivity in animal models of opiate and alcohol dependence, suggesting a possible emergence of hyperalgesia that may relate to excessive drug intake in dependent animals. Opiates and alcohol activate similar neuroanatomical and molecular systems (Koob and Bloom, 1988; Herz, 1997; Koob et al., 2003; Siggins et al., 2003; Modesto-Lowe and Fritz, 2005; Gianoulakis, 2009), and also share a close similarity with regard to withdrawal symptomatology (West and Gossop, 1994). In addition, mice that display an alcohol deprivation effect (elevated drinking levels as a consequence of withdrawal) also display greater thermal hyperalgesia during concomitant morphine withdrawal (Salimov et al., 1993). However, there remains a gap in our knowledge regarding how specific negative emotional states that precede or develop during chronic drug use influence compulsive drug-seeking behaviors. A link between hyperalgesia and an enhanced motivation to obtain opiates has been demonstrated in rats (Martin et al., 2007). In animals with reduced paw withdrawal thresholds following spinal nerve ligation, only heroin doses that effectively produced alleviation of hyperalgesia maintained opiate self-administration, whereas lower doses were only effective in maintaining opiate self-administration in control (sham-operated) rats, suggesting that the driving force for the motivation to self-administer drugs in individuals with a sensitized nociceptive system may be in part to seek relief of chronic pain. Nerve-injured rats would seem to require higher doses of heroin to

register reward, and are also more sensitive to mu-opioid receptor blockade (Martin et al., 2011).

In our study, paw withdrawal thresholds during heroin withdrawal were significantly and negatively correlated with individual, preferred levels of heroin intake after two weeks of extended access self-administration (Figure 4-2C). This relationship may represent a direct dose-response between heroin intake and resultant mechanical hypersensitivity, but could also indicate that animals more sensitive to tactile stimulation self-administered more heroin to overcome this condition. In contrast to heroin-dependent animals, we did not detect paw withdrawal threshold changes in either cocaine LgA or ShA rats (Figure 4-3). Cocaine is an analgesic (Altier and Stewart, 1999; Pamplona et al., 2007) that activates both opponent motivational processes (Ettenberg, 2004) and endogenous opioid systems (Kreek, 1996; Roth-Deri et al., 2003; Boutrel, 2008), although endogenous opioid signaling may play a more direct role in incentive sensitization mechanisms related to cocaine-seeking behaviors (Self, 2004; Simmons and Self, 2009). A recent study found that induction of a chronic pain-like state induced by spinal nerve ligation was able to block morphine-induced, but not cocaine-induced, potentiation of rewarding electrical brain stimulation (Ewan and Martin, 2011), suggesting a lack of association between exaggerated nociception and cocaine reward in line with results from the present study.

In accordance with prior reports (e.g., Dina et al., 2000), protracted exposure to dependence-inducing ethanol concentrations resulted in a lowering of nociceptive thresholds (Figure 4-4B). Dina and colleagues have further

characterized the relationship between ethanol exposure patterns and the establishment of neuropathy-like conditions (Dina et al., 2006; Dina et al., 2007). The results of these studies indicate that enhanced mechanosensitivity appears more rapidly by increasing the withdrawal periods between alcohol exposures in what the authors considered a model of binge-like drinking. They also discovered that the hypersensitivity persists or is even exacerbated during withdrawal, consistent with human data (e.g., Yokoyama et al., 1991). Finally, Dina et al. (2008) implicated glucocorticoid receptor signaling in the development of alcohol withdrawal-induced reduction of thresholds. The present study extends these findings by implicating another critical component of stress-related signaling, the CRF<sub>1</sub> receptor, in the maintenance of enhanced mechanosensitivity in ethanol-dependent animals. Indeed, it is well documented that glucocorticoids can sensitize extra-hypothalamic CRF systems (Imaki et al., 1991; Makino et al., 1994; Shepard et al., 2000), although future studies are required to delineate central vs. peripheral actions of CRF signaling on drug-induced mechanical hypersensitivity.

#### *Corticotropin-Releasing Factor, Pain, and Drug Dependence*

The role of CRF in pain signaling is complex, and CRF typically interacts with opioid peptide signaling at both central and peripheral nervous system components (Mousa et al., 2007). In the periphery, CRF, potentially acting via both CRF<sub>1</sub> and CRF<sub>2</sub> receptors (Mousa et al., 2003), can also directly activate immune cells that have accumulated at injured peripheral nerves to produce



antinociception via opioid-dependent mechanisms (Schafer et al., 1997; Labuz et al., 2006; Labuz et al., 2010). Early studies also described an antinociceptive role of CRF in the context of stress-induced analgesia (for review, see Lariviere and Melzack, 2000), although CRF produced hyperalgesic effects in models of visceral hypersensitivity (Tache et al., 2005). More recent studies have attempted to reconcile these differences, with significant progress. For example, Lariviere et al. (2011) revealed differential effects of central CRF on distinct behavioral responses in the formalin test. Additionally, with the development of more selective pharmacological tools for distinguishing specific CRF receptor-subtype function, recent work suggests that the central anti-nociceptive effects of CRF are most likely mediated through CRF<sub>2</sub> receptors (Ji and Neugebauer, 2008). In contrast, CRF<sub>1</sub> receptors mediate the pro-nociceptive effects of this peptide, and this relationship is mediated at least partly via the central amygdala (Ji and Neugebauer, 2007; Fu and Neugebauer, 2008). CRF<sub>1</sub> receptors also mediate pain-related anxiety-like behavior (Ji et al., 2007). At the intracellular level, several recent studies have delineated a critical role for amygdala extracellular signal-regulated kinase (ERK) signaling (Carrasquillo and Gereau, 2007; Fu et al., 2008) in pain-associated synaptic plasticity and behavior. ERK phosphorylation in the amygdala is increased during withdrawal from either escalated heroin self-administration (Edwards et al., 2009) or after ethanol vapor-induced dependence induction (Sanna et al., 2002), while selective CRF<sub>1</sub>R antagonism reduces both ethanol (Richardson et al., 2008) and heroin self-administration (Greenwell et al., 2009) in dependent animals. Thus, enhanced

CRF<sub>1</sub>R-ERK signaling in the amygdala represents one possible molecular node of intersection between pain-related negative affect and drug addiction.

The antinociceptive effects of CRF<sub>1</sub>R antagonists have been demonstrated across several pain models, although this class of drugs does not alter various pain-related indices (e.g., audible or ultrasonic vocalizations, paw withdrawal thresholds) in non-injured animals (e.g., Fu and Neugebauer, 2008). Similarly, CRFR antagonism does not alter pain-related behavior in non-dependent animals in the tail flick test (McNally and Akil, 2002) or in any non-dependent model in terms of paw withdrawal thresholds in the present study. Since mechanical hypersensitivity is produced by chronic drug exposure (in the case of opiates and alcohol) and since we hypothesize that this state drives renewed drug intake as a negative reinforcement mechanism, we believe that CRF<sub>1</sub>R antagonism represents a viable therapeutic strategy targeting this condition.

In the present study, mechanical hypersensitivity was observed during spontaneous heroin withdrawal (i.e., without opioid receptor antagonist administration), a condition hypothesized to be associated with a sensitization of pronociceptive systems following chronic heroin exposure (Celerier et al., 2001). In comparison to opiate-induced nociceptive sensitization via descending facilitation following continuous drug exposure (e.g., Vanderah et al., 2001; Ossipov et al., 2004) we feel that our observations better model an opiate withdrawal-induced state of hypersensitivity (Gutstein, 1996) whereby neurotransmitter signaling mechanisms opponent to mu-opioid receptor

stimulation (including CRF signaling) are recruited and subsequently unmasked on an intermittent (daily) basis during withdrawal selectively in LgA animals. Based on our daily observations, in addition to reduced paw withdrawal thresholds, heroin LgA animals readily exhibit a host of spontaneous physical withdrawal symptoms prior to self-administration including excessive grooming, diarrhea, and vocalization upon handling (Vendruscolo et al., 2011). These symptoms emerge in parallel with the manifestation of brain reward deficits (Kenny et al., 2006) and enhanced heroin-seeking behavior (Lenoir and Ahmed, 2007) observed during withdrawal in rats given extended access to heroin self-administration. Attempts to alleviate such physical and/or motivational withdrawal states may underlie compulsive heroin seeking, while tolerance to the antinociceptive and other positive rewarding effects of opiates may in part drive the need to escalate heroin intake across days in LgA rats.

A role for amygdala CRF receptor signaling in regulating thermal hyperalgesia following precipitated opiate withdrawal in morphine-dependent animals was previously demonstrated by McNally and Akil (2002). In this study, rats were made dependent via morphine pellet and withdrawal was precipitated by naloxone. Under these conditions, dependent rats exhibited a reduced tail flick latency compared to sham-pelleted, naltrexone-treated animals, and this thermal hyperalgesia was alleviated by microinjection of the non-selective CRF receptor antagonist alpha helical CRF<sub>9-41</sub> into the central amygdala (but not the bed nucleus of the stria terminalis). Thus, CRF would appear to play a role in the expression of both mechanical and thermal hypersensitivity during opiate

dependence. Importantly, administration of a CRF receptor antagonist into the CeA also reduces the reward associated with negative reinforcement during morphine withdrawal (Heinrichs et al., 1995), further suggesting possible links among negative affect, hyperalgesia, and CRF signaling after chronic opiate exposure.

One remaining unexplored issue is how extended periods of withdrawal influence paw withdrawal thresholds. Ethanol-dependent rats display heightened anxiety-like behavior up to four weeks into withdrawal (Valdez et al., 2002). The ability of naltrexone to precipitate hyperalgesia in heroin-exposed animals is also maintained for up to 25 days following discontinuation of heroin (Celerier et al., 2001). Ren et al. (2009) found that a hyperalgesic state can persist for up to five months in abstinent opiate addicts, while addicts with more pain sensitivity also displayed greater cue-induced craving at this time point. Further investigation of the effects of persistent pain-like states in the context of long-term self-administration and drug-seeking behaviors is warranted for the benefit of both pain and addiction fields (Barrot, 2011).

## **CONCLUSIONS**

This study revealed a differential ability of three common drugs of abuse to alter paw withdrawal thresholds, with chronic heroin and alcohol (but not cocaine) producing a significant mechanical hypersensitivity that could represent part of a negative emotional state associated with excessive drug exposure. Pharmacological blockade of CRF<sub>1</sub> receptors partially alleviated this

hypersensitivity, which may in part explain the efficacy of CRF<sub>1</sub>R antagonists in reducing excessive heroin and ethanol self-administration in dependent animals. Since a magnified CRF<sub>1</sub> receptor signaling appears to be linked to chronic pain-related mechanisms and elevated heroin and alcohol intake, CRF<sub>1</sub>R antagonism, by attenuating the negative motivational effects of mechanical hypersensitivity during drug withdrawal, may represent a viable therapeutic strategy for ethanol- and opiate-dependent individuals, particularly those suffering from hyperalgesia.

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Chapter 4, in full, is a formatted reprint of the material as it appears in *Neuropharmacology*, 2012. Edwards S, Vendruscolo LV, Schlosburg JE, Misra K, Wee S, Park PE, Schulteis G, Koob GF. The dissertation author was a coauthor of this paper.

## **CHAPTER 5**

### **Chronic CRF<sub>1</sub> receptor blockade reduces heroin intake escalation and dependence-induced hyperalgesia**



**ABSTRACT**

Opioids represent effective drugs for the relief of pain, yet chronic opioid use often leads to a state of increased sensitivity to pain that is exacerbated during withdrawal. A sensitization of pain-related negative affect has been hypothesized to closely interact with addiction mechanisms. Neuroadaptive changes occur as a consequence of excessive opioid exposure, including a recruitment of corticotropin-releasing factor (CRF) and norepinephrine (NE) brain stress systems. To better understand the mechanisms underlying the transition to dependence, we determined the effects of functional antagonism within these two systems on hyperalgesia-like behavior during heroin withdrawal utilizing models of acute and chronic dependence. We found that passive or self-administered heroin produced a significant mechanical hypersensitivity. During acute opioid dependence, systemic administration of the CRF1 receptor antagonist MPZP (20 mg/kg) alleviated withdrawal-induced mechanical hypersensitivity. In contrast, several functional adrenergic system antagonists (clonidine, prazosin, propranolol) failed to alter mechanical hypersensitivity in this state. We then determined the effects of chronic MPZP or clonidine treatment on extended access heroin self-administration and found that MPZP, but not clonidine, attenuated escalation of heroin intake, whereas both drugs alleviated chronic dependence-associated hyperalgesia. These findings suggest that an early potentiation of CRF signaling occurs following opioid exposure that begins to drive both opioid-induced hyperalgesia and intake escalation.

## INTRODUCTION

Drug addiction is a chronically relapsing disorder characterized by drug intake escalation and the emergence of negative emotional states during abstinence (Edwards and Koob, 2010). A negative emotional state is defined as a dysphoric state accompanied by depression- and anxiety-like symptoms and is thought to contribute to the compulsivity associated with dependence via the process of negative reinforcement (Koob, 2008). A sensitization of negative affect associated with pain systems (Ji et al., 2007) has also been hypothesized to be mediated by central reinforcement circuitry and to closely interact with addiction mechanisms (Shurman et al., 2010; Egli et al., 2012).

Approximately one third of the adult population in the United States suffers from chronic pain (Johannes et al., 2010). Many different formulations of opioids have been proven to be effective in the treatment of a variety of chronic pain conditions, and opioids are one of the best resources for analgesia and the improvement in quality of life. Although only a small percentage of chronic pain patients using opioids become addicted (Fishbain et al., 2008), abuse of and addiction to opioids in vulnerable populations continue to be a major health concern. Chronic opioid exposure for the purpose of alleviating pain often renders individuals more sensitive to nociception, a condition known as hyperalgesia (Angst and Clark, 2006), which may be associated with the transition to dependence in patients with abuse histories. Hyperalgesia is prevalent in former opioid addicts who are maintained on methadone as a treatment of opioid dependence, suggesting that this condition emerges with

extended opioid use (Compton et al., 2001). Opioid exposure in rodents leads to a spontaneous reduction in mechanical nociceptive thresholds (Laulin et al., 1998) that is exacerbated after chronic treatment (Simonnet and Rivat, 2003), suggesting a recruitment or sensitization of pro-nociceptive systems (Celerier et al., 2001). Given that chronic pain is well known to cause emotional distress and produce a sustained negative emotional state (King et al., 2009), opioid-induced hyperalgesia may constitute a condition intimately associated with the progressive transition to drug dependence by facilitating negative reinforcement processes. Characterizing pain responsiveness during both acute and chronic opioid administration may extend our knowledge of the early processes promoting negative reinforcement mechanisms associated with the gradual transition to addiction.

Opioid addiction has been linked to neuroadaptation and dysregulation of brain stress systems (Edwards et al., 2009; Koob, 2008), including those regulated by corticotropin-releasing factor (CRF) and norepinephrine (NE) within the extended amygdala (Heinrichs et al., 1995; Koob and Le Moal, 2008). Extracellular CRF in the extended amygdala is increased during acute withdrawal from opioids, and CRF receptor antagonists block excessive heroin intake associated with dependence (Greenwell et al., 2009; Weiss et al., 2001). Importantly, CRF<sub>1</sub> receptor antagonism dose-dependently alleviates mechanical hypersensitivity exhibited by opioid-dependent rats (Edwards et al., 2012; McNally and Akil, 2002). Similarly, NE in the extended amygdala and locus coeruleus increases during acute withdrawal from drugs of abuse, and

hyperactivity of brain NE accompanied by increased CRF signaling has been implicated in mechanisms of opioid withdrawal in the extended amygdala (Aston-Jones et al., 1999; Maldonado, 1997; Smith and Aston-Jones, 2008). Interestingly, intrathecal clonidine (a presynaptic alpha 2-adrenoceptor agonist) has been shown to reverse mechanical hyperalgesia and reduce heroin intake only in spinal nerve-injured rats (Martin et al., 2007). To further investigate the interactive role of nociception and brain stress systems during opioid withdrawal (Shurman et al., 2010), we wanted to determine the effects of CRF and NE signaling on pain- and addiction-like behaviors utilizing models of acute and chronic opioid dependence.

## **MATERIALS AND METHODS**

### *Animals*

Male Wistar rats (n = 79) weighing between 200 - 300 g were housed in groups of two to three per cage, and maintained on a 12-h light/dark cycle (lights on at 0800) with free access to food and water. The animals were allowed to acclimate to these conditions for at least 7 days in our animal facilities before behavioral testing. Animals were regularly handled for one week prior to surgery or any behavioral testing. All procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

### *Mechanical Sensitivity Testing*

This test was conducted as previously reported (Edwards et al., 2012). Up to eight rats were placed in individual plastic compartments (26 x 11 x 20 cm) with stainless steel mesh floors for 30 minutes until the rats' grooming and exploratory behaviors ceased. To assess the presence of mechanical hypersensitivity, the mid-plantar area of each hind paw was perpendicularly stimulated with calibrated nylon von Frey filaments (Weinstein-Semmes algometer forces) for 5 seconds using the up-down method starting with the 28.84 g force. A brisk withdrawal of the paw (often followed by a sustained retraction and/or licking, possibly indicative of supraspinal organization) is considered a positive response, but paw withdrawals due to locomotion or weight shifting were not counted. For quantitative assessment, the 50% probability withdrawal threshold, or paw withdrawal threshold (PWT), was calculated as previously described (Chaplan et al., 1994). Baseline mechanical nociceptive thresholds were similar to those reported for the ages of rats employed in this study (Ririe and Eisenach, 2006).

### *Drugs*

Heroin (3,6-diacetylmorphine) was provided by the National Institute on Drug Abuse and was dissolved in 0.9% sterile saline and injected subcutaneously (SC). Clonidine hydrochloride (presynaptic alpha-2 adrenoceptor agonist) was purchased from Sigma-Aldrich and dissolved in 0.9% saline and injected SC in a volume of 1 ml/kg body weight. Prazosin hydrochloride (alpha-1

adrenoceptor antagonist) and propranolol hydrochloride were purchased from Sigma-Aldrich and dissolved in 0.9% saline and injected intraperitoneally (IP) in a volume of 1 ml/kg body weight. The CRF 1 receptor antagonist MPZP was prepared for systemic administration as described (Richardson et al., 2008). Animals were administered MPZP in a volume of 2 ml/kg 20% HBC (SC). For the chronic prophylactic administration studies, the vehicle-treated rats were given repeated SC injections of 2 ml 20% HBC vehicle/kg body weight.

#### *Acute heroin dependence model*

Acute opioid dependence models are designed to reveal early behavioral neuroadaptations associated with the initiation and progression of dependence symptomatology (Azar et al., 2003; Liu and Schulteis, 2004; Schulteis et al., 1999; Zhang and Schulteis, 2008). To model acute heroin dependence, animals were injected (SC) daily with 1.25 mg/kg heroin. This dose was previously shown to induce mechanical hyperalgesia during heroin withdrawal (Laulin et al., 1998) that progressively increases after repeated, intermittent heroin injections (Celerier et al., 2001). Control animals received repeated injections of saline on equivalent schedules.

#### *Heroin self-administration*

The surgery and self-administration procedures have been reported in detail previously (Vendruscolo et al., 2011). Briefly, rats were anesthetized with isoflurane (2%) and chronic intravenous catheters were placed in the jugular

vein. Rats were allowed to recover for 7 days before behavioral testing. Rats were trained to lever press for heroin (60 µg/kg/infusion) 1 h per day, on a fixed-ratio (FR) 1 schedule, 5 days per week. Drug infusion was paired with a cue light (above the active lever) for 20 s signaling a time-out period. Presses during the time-out period were recorded but no drug was delivered. Once stable lever press responding was achieved, the rats were split in two groups matched for responding (baseline): short-access (1 h session: ShA) or long-access (12 h session: LgA), to test mechanical sensitivity and intake. For the prophylactic drug regimen study (Fig. 4), rats were split into three pretreatment groups with 8 h long-access sessions. During all self-administration sessions, rats were allowed to nose-poke for food (45 mg pellets, Bio-Serve) on an FR 3 schedule, and water under an FR 1 schedule.

### *Pharmacological Testing*

All pretreatment times and doses were derived from published studies (see discussion). For the acute dependence studies, rats were injected with heroin or saline 24 h prior to testing, and then pretreated with drugs or vehicles at different time points. Clonidine (10 µg/kg) or MPZP (20 mg/kg) was given 60 min prior to paw withdrawal testing. Prazosin (2 mg/kg) or propranolol (10 mg/kg) was given 30 min prior to paw withdrawal testing. For the self-administration study, vehicle (20% HBC), clonidine (10 µg/kg), or MPZP (20 mg/kg) was injected SC immediately prior to each self-administration session.

### *Statistical Analysis*

All data are expressed as means and standard errors of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) with group (repeated saline, repeated heroin) as a between-subjects factor or using a repeated-measure two-way ANOVA with group as a between-subjects factor and treatment (clonidine, MPZP, prazosin, or propranolol vs. vehicle) as within-subjects factors. Fisher's Least Significant Difference (LSD) tests or Dunnett's tests were used for *post hoc* comparisons when appropriate. All statistical analyses were performed with Prism 6.0 or Statistica 10, and p-values less than 0.05 were considered statistically significant.

## **RESULTS**

### **Acute heroin dependence induces mechanical hypersensitivity**

Figure 5-1A shows the development of mechanical hypersensitivity following a series of intermittent heroin injections (1.25 mg/kg, SC). Rats were tested 1, 3, and 6 h after heroin injections on day 1 and day 5, in addition to 24 h after daily heroin administration for five days. Comparing the heroin-treated rats between day 1 and day 5, the ANOVA revealed an overall group effect ( $F_{(1, 14)} = 19.09$ ,  $p < 0.001$ ) as well as a time effect ( $F_{(3, 42)} = 51.2$ ,  $p < 0.0001$ ). Thus, heroin produced a significant modification of paw withdrawal thresholds that shifted downward over days, indicative of a sensitization of pronociceptive systems (Celerier et al., 2001). Figure 5-1B depicts the lowering of paw withdrawal thresholds over several days of repeated heroin administration. One-



way ANOVA revealed a day effect for heroin ( $F_{(4, 24)} = 5.03, p < 0.005$ ). *Post hoc* comparisons showed a significant difference on day 4 and 5 compared to day 1 ( $p < 0.05$ ). No effect was seen for the vehicle group ( $F_{(4, 28)} = 1.15, p = 0.35$ ).

### **Paw withdrawal thresholds decrease as heroin intake escalates**

Figure 5-2 represents the development of hyperalgesia during heroin self-administration. Heroin intake data (A) show that LgA rats increase the number of heroin infusions while the ShA rats maintain a low, stable number of infusions. The ANOVA revealed a group  $\times$  session interaction:  $F_{(13, 130)} = 2.12, p < 0.05$ . Dunnett's post-hoc analysis showed a significant escalation of intake in the LgA group starting on day 11 ( $p < 0.05$ ) until day 14 ( $p < 0.01$ ) compared to day 1. We tracked mechanical sensitivity in parallel with heroin intake escalation (B). The ANOVA revealed an overall group effect between ShA and LgA ( $F_{(1, 11)} = 4.86, p < 0.05$ ) and a day effect ( $F_{(5, 55)} = 7.55, p < 0.0001$ ). On day 1, both ShA and LgA rats tended to show a decrease in paw withdrawal thresholds. After day 1, however, thresholds for ShA rats become stable, whereas those for LgA rats continue to decrease. By day 14, the thresholds for ShA rats significantly differ from those of LgA rats ( $p < 0.005$ ).

### **MPZP reverses acute heroin dependence-induced mechanical hypersensitivity**

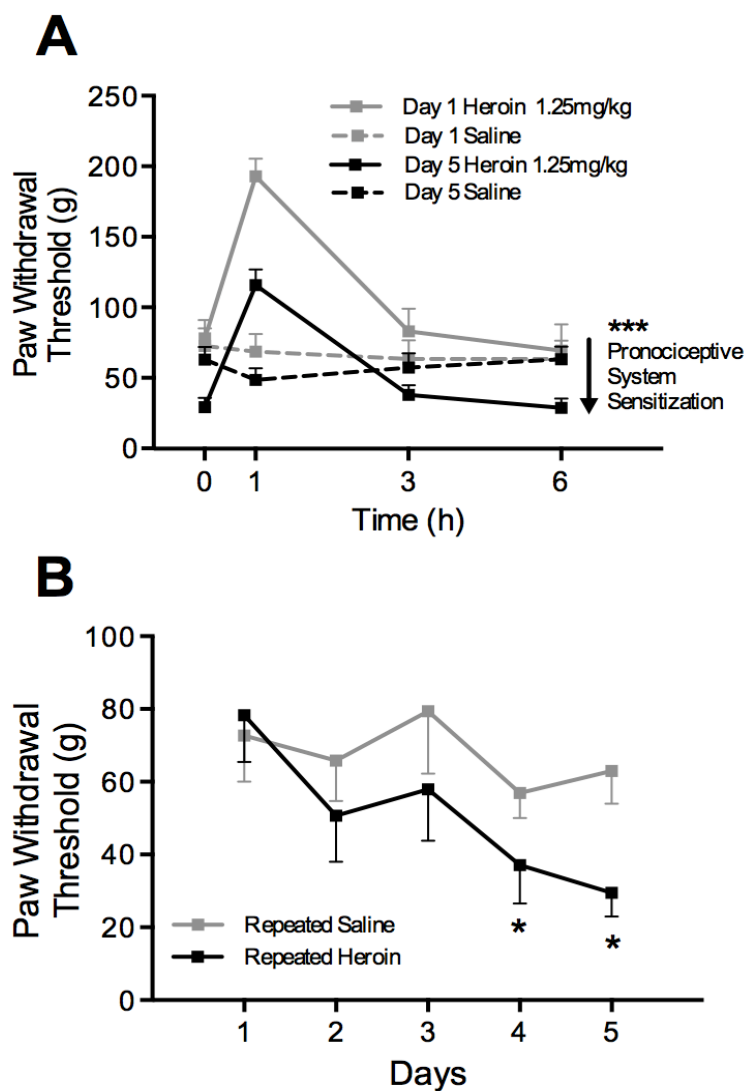
Figure 5-3A represents the effects of MPZP on heroin-induced decreases in paw withdrawal thresholds. MPZP significantly alleviated the hyperalgesia

during acute dependence. The ANOVA revealed a treatment  $\times$  group interaction ( $F_{(1, 14)} = 6.13, p < 0.05$ ). Post-hoc analysis revealed that the heroin-vehicle group differed from the heroin-MPZP group ( $p < 0.05$ ) and that the heroin-vehicle group was different from the saline-vehicle group ( $p < 0.0001$ ). Fig 5-3B shows the effect of clonidine at two different doses on paw withdrawal thresholds during acute heroin dependence. The ANOVA revealed only a main heroin effect for clonidine at doses of 10  $\mu\text{g}$  ( $F_{(1, 14)} = 16.82, p < 0.005$ ) or 50  $\mu\text{g}$  ( $F_{(1, 14)} = 42.00, p < 0.0001$ ). Fig 5-3C shows that prazosin did not reverse the lowered (main effect of heroin:  $F_{(1, 14)} = 13.89, p < 0.005$ ) paw withdrawal thresholds during acute heroin dependence. Fig 5-3D shows the effect of propranolol on paw withdrawal thresholds during acute dependence. Although there seems to be a slight reversal of the heroin-lowered thresholds, the ANOVA revealed only the factor of heroin exposure significantly lowering thresholds ( $F_{(1, 12)} = 17.65, p < 0.005$ ).

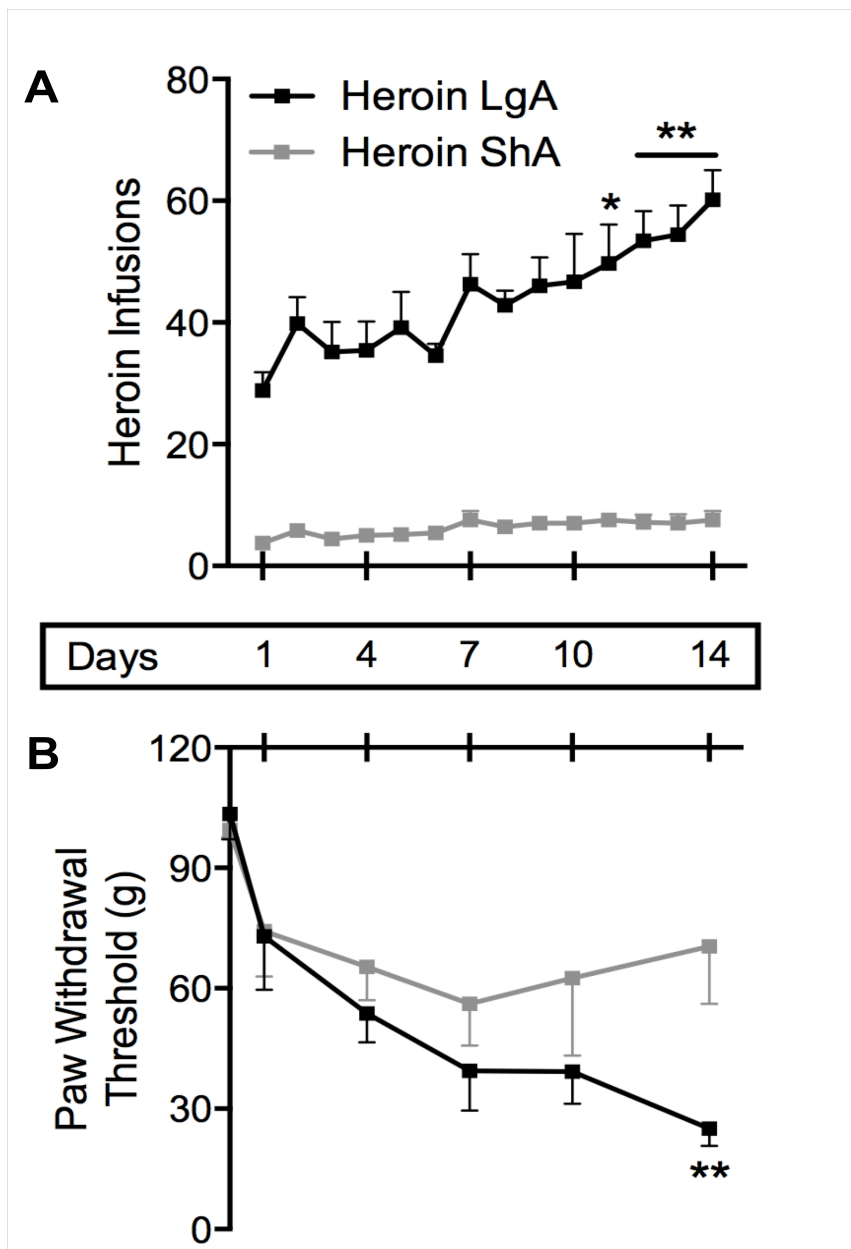
### **MPZP attenuates escalation of heroin self-administration and hyperalgesia**

Figure 5-4 shows the percent change from chronic vehicle-treated animals on treatment day one. Rats were trained to lever press for heroin and split into three groups ( $n = 6$ ) according to the first long access (8hr) session intake (baseline session). A pretreatment of vehicle, MPZP, or clonidine was given prior to each self-administration treatment session for a total of 14 treatment sessions. Figure 5-4A represents the % change in heroin intake for rats treated with MPZP or vehicle. The ANOVA revealed an interaction between group and day ( $F_{(13, 130)} = 2.63, p < 0.005$ ). Post-hoc analyses indicated that vehicle-treated rats

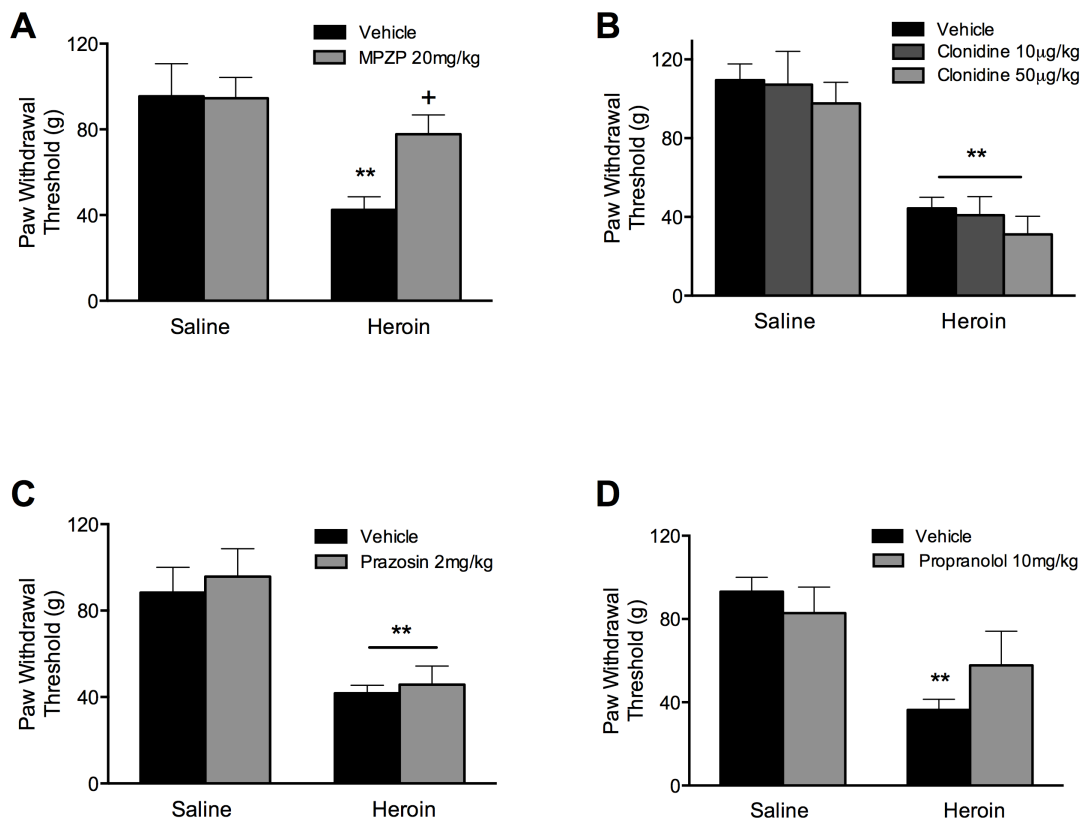
exhibited a significant increase in heroin intake compared to MPZP-treated rats on days 10, 13, and 14 ( $p < 0.05$ ). Intake during the first hour of each treatment sessions is shown in Figure 5-4B. Rats treated chronically with MPZP also displayed significantly higher paw withdrawal thresholds ( $t_{10} = 2.642$ ,  $p < 0.05$ ; C). Figure 5-4 D-E shows the comparison between the same vehicle group and the chronic clonidine group. No significant differences were observed in heroin intake over time, although a trend for decreased heroin intake during the first hour of self-administration was observed (main effect of group,  $F_{(1,10)} = 3.59$ ,  $p = 0.09$ ). Despite similar levels of heroin intake, animals treated chronically with clonidine displayed significantly higher paw withdrawal thresholds ( $t_{10} = 5.232$ ,  $p < 0.001$ ; F).



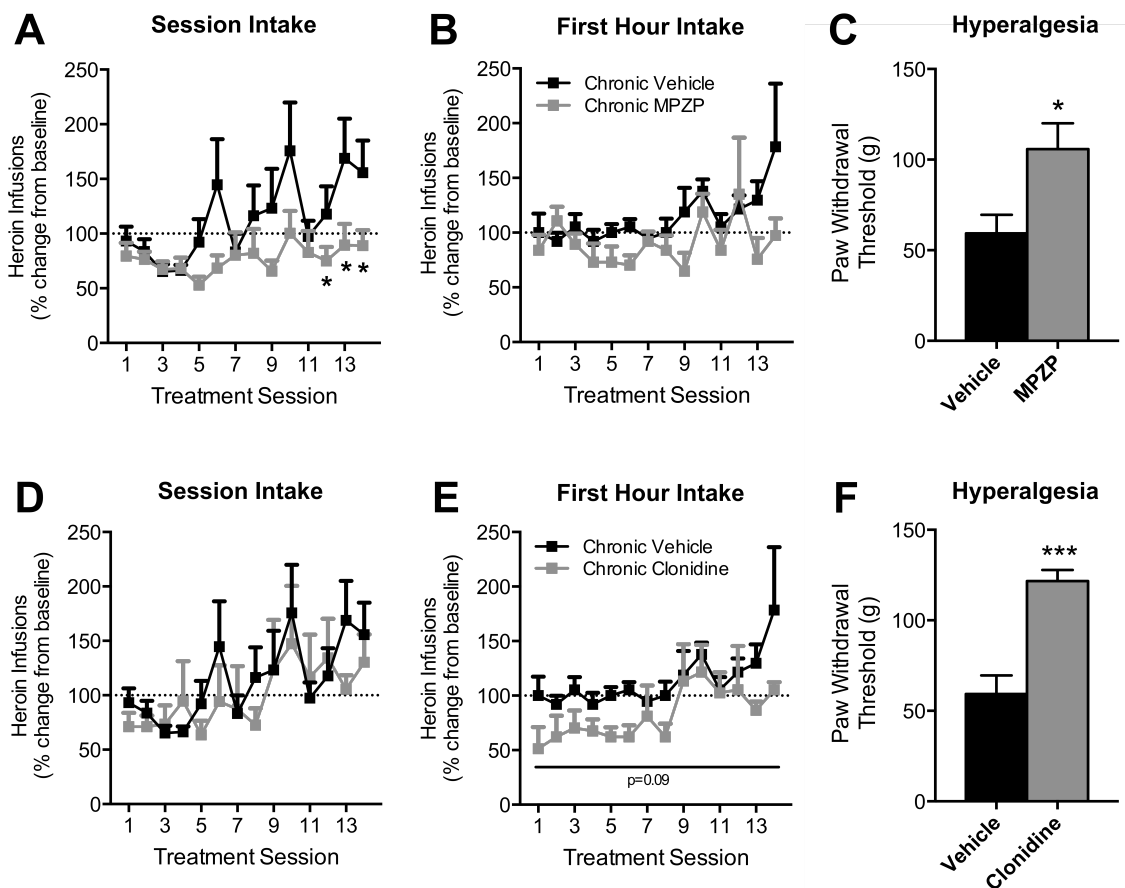
**Figure 5-1. (A)** Mechanical sensitivity response after heroin injection. Rats were administered heroin (1.25 mg/kg, SC) for five days and tested for paw withdrawal thresholds 1, 3, 6 h post-heroin injection on day 1 and day 5. The magnitude of the increase in threshold at 1 h is lessened on day 5, while the thresholds at 0 h and 6 h are below day 1, indicating hyperalgesia. These neuroadaptations are hypothesized by Simonnet and colleagues (Celerier et al., 2001) to reflect a sensitization of pronociceptive systems. Asterisks indicate a significant group effect (\*\* $p < 0.01$ ) between acute vs. chronic heroin-treated groups. **(B)** Paw withdrawal thresholds of heroin-treated rats continue to decrease over five days. On days 4 and 5, thresholds are significantly lower compared to baseline (\*  $p < 0.05$ ).



**Figure 5-2. (A)** Gradual development of mechanical hyperalgesia across heroin self-administration history. Paw withdrawal thresholds were measured in heroin self-administering rats over the escalation period. **(B)** As long-access (LgA) rats increase their intake of heroin, thresholds continue to decrease over time. Heroin intake for LgA rats increased significantly compared to baseline starting on day 11 (\*  $p < 0.05$ ), and paw withdrawal thresholds were different from short-access (ShA) rats on day 14 (\*\*  $p < 0.01$ ). Paw withdrawal thresholds of ShA rats do not decrease significantly compared to baseline, and their heroin intake remains relatively constant.



**Figure 5-3.** CRF<sub>1</sub>R antagonism, but not functional adrenergic receptor antagonism, reverses heroin withdrawal-induced mechanical hypersensitivity during acute dependence. For all tests, heroin-treated rats showed significantly lower paw withdrawal thresholds compared to saline-treated rats (\*\*  $p < 0.005$ ). **(A)** MPZP treatment significantly raised thresholds, indicating an alleviation of mechanical hypersensitivity (<sup>+</sup>  $p < 0.05$ ). **(B-D)** Neither clonidine (10 µg/kg, 50 µg/kg, SC), prazosin (2mg/kg, IP), nor propranolol (10 mg/kg, IP) alleviated the acute heroin dependence-induced mechanical hypersensitivity.



**Figure 5-4.** Heroin intake expressed as percent change from chronic vehicle-treated animals on treatment day one and paw withdrawal thresholds measured at the end of the study. Rats were trained to lever press for heroin and split into three groups ( $n = 6$  each) receiving repeated treatments of vehicle, MPZP, or clonidine prior to each self-administration session. Over 14 treatment sessions, vehicle-treated rats displayed a gradual increase in heroin intake that significantly diverged from the chronic MPZP-treated rats by the end of the training (**A-B**). A significant difference between vehicle and MPZP groups was present on days 10, 13, and 14. (\*  $p < 0.05$ ). Animals treated chronically with MPZP also displayed higher paw withdrawal thresholds, reflecting an absence of heroin-induced hyperalgesia (**C**). In comparison, heroin self-administering animals treated chronically with clonidine did not display a significant difference in intake from the vehicle group across the entire eight-hour session (**D**) but did exhibit a trend for decreased heroin intake over the first hour of self-administration (**E**). Despite similar heroin intake levels between groups, clonidine-treated animals exhibited higher paw withdrawal thresholds (**F**), suggesting a reduction of heroin-induced hyperalgesia in this group relative to animals receiving chronic vehicle treatments.

## DISCUSSION

In accordance with previous reports, we found a significant mechanical hypersensitivity associated with both acute heroin dependence and heroin intake escalation. The CRF<sub>1</sub> receptor antagonist MPZP alleviated mechanical hypersensitivity in the acute dependence model, further confirming the role of this drug class in alleviating hyperalgesia resulting from either injury (Ji and Neugebauer, 2007) or chronic exposure to opioids (Edwards et al., 2012; McNally and Akil, 2002).

In contrast to CRF<sub>1</sub>R antagonism, a variety of drugs systemically blocking adrenergic neurotransmission did not significantly alter paw withdrawal threshold reductions associated with limited, passive heroin exposure (modeling acute dependence). This represents a significant dissociation from the observed anti-hyperalgesic effects of adrenoceptor blockade across multiple pain models. For example, systemic administration of prazosin (2 mg/kg) alleviates subcutaneous hindpaw bee venom-induced mechanical hyperalgesia (Chen et al., 2010), while even lower doses are effective against cold allodynia in a neuropathic pain model (Kim et al., 2005). Chronic administration of a lower dose (0.3 mg/kg) of prazosin delayed the development of both streptozotocin- and vincristine-induced hyperalgesia, models of diabetic and toxic neuropathy, respectively (Bujalska et al., 2008). Similarly, doses of propranolol identical to or lower than that employed here (i.e., 1-10 mg/kg) alleviate pain hypersensitivity following catechol-O-methyltransferase inhibition (Nackley et al., 2007) or intraplantar endotoxin administration (Safieh-Garabedian et al., 2002). Our data showed a



trend for a reversal of acute heroin-induced mechanical hypersensitivity with 10 mg/kg propranolol. The expression of chronic opioid-induced hyperalgesia has been tied to beta-2-adrenergic receptor activity (Liang et al., 2006), and this receptor has also been linked to opioid tolerance and chronic dependence (Liang et al., 2007), suggesting that potentiation of this system may occur in response to repeated opioid exposure. In addition, it was recently found that hyperalgesia induced by termination of remifentanil exposure in humans was attenuated by concurrent propranolol exposure (Chu et al., 2012), suggesting that beta-adrenoceptor blockade during opioid exposure may be more effective in reducing the resultant hyperalgesia.

Alpha-2 adrenergic agonists such as clonidine possess valuable analgesic activity, reduce postoperative opioid consumption (Blaudszun et al., 2012), but also display a narrow therapeutic window and can produce significant side effects (Martin and Eisenach, 2001). Thermal- or capsaicin-induced pain relief occurs following intrathecal, but not intravenous, clonidine administration in humans (Eisenach et al., 1998), highlighting the potential importance of administration route. Interestingly, in rats, spinal clonidine administration also produces a conditioned place preference (a model of reward) only in animals experiencing pain (Davoody et al., 2011; He et al., 2012; King et al., 2009). For the present study, we administered relatively low clonidine doses below the presumptive analgesic dose range. Indeed, neither the doses of clonidine nor any dose of the other compounds examined increased paw withdrawal thresholds in saline-treated animals, which likely represents an absence of

analgesic or motor-disruptive effects typically observed following acute administration of opioid analgesics (e.g., Stowe et al., 2011). Instead, clonidine appears to regulate opioid withdrawal-related behaviors at the doses tested (10-50 µg/kg). For example, clonidine (50 µg/kg) reduces naloxone-precipitated physical withdrawal symptoms after chronic morphine exposure (Tierney et al., 1988). Clonidine administered at 10-40 µg/kg also blocks stress-induced reinstatement to heroin-seeking behavior (Shaham et al., 2000). However, this range of dosing failed to alleviate acute heroin dependence-induced hyperalgesia, suggesting that potentiation of this receptor system may occur only after extensive or excessive opioid exposure to regulate pain-related behaviors (see below).

Previous studies have found that noradrenergic receptor blockade reduces both anxiety-like behavior (Park et al., 2013) and heroin intake in dependent rats for the first hour of extended access (Greenwell et al., 2009). Both CRF and NE increase in the extended amygdala during drug withdrawal (Maldonado, 1997; Weiss et al., 2001) and activation of this brain stress system is thought to mediate the negative emotional states that manifest during opioid withdrawal. It is thought that a CRF-NE feed forward brain stress system exists where CRF from the extended amygdala activates brainstem NE activity and, in turn, NE activates forebrain CRF activity (Koob, 1999). The effects of CRF in this brain stress system during opioid withdrawal seem consistent throughout different behavioral measures, as CRF has shown to potentially drive NE during acute heroin withdrawal, while the CRF<sub>1</sub>R antagonist MPZP blocked withdrawal-

potentiated startle (Park et al., 2013). However, unlike what we observed with regard to hyperalgesia-like behavior here, adrenergic blockers have been shown to decrease opioid withdrawal-potentiated startle in rats (Harris and Gewirtz, 2004; Park et al., 2013; Rothwell et al., 2009), representing a significant dissociation between pain- and anxiety-like behaviors, along with differences in the neuropharmacological mechanisms that mediate these states during opioid withdrawal. Our results also appear to suggest unique roles for adrenergic receptor signaling across different pain models (i.e., injury vs. drug-induced hyperalgesia), and further comparative exploration in these areas is needed.

Chronic, prophylactic administration of MPZP (20 mg/kg) attenuated escalation of heroin self-administration, indicating a possible link between CRF and early neuroadaptive mechanisms associated with the eventual transition to heroin addiction. CRF<sub>1</sub>R antagonist-mediated decreases in heroin intake in LgA rats are thought to occur due to the attenuation of negative reinforcement processes that accompany LgA heroin intake (Chen et al., 2006; Greenwell et al., 2009; Kenny et al., 2006; Koob et al., 2004). In contrast, chronic treatment with clonidine (10 ug/kg) failed to alter heroin intake over the eight-hour sessions, although there was a trend for a reduction in intake during the first hour of self-administration. Not surprisingly, animals treated chronically with MPZP (who self-administered less heroin) exhibited higher paw withdrawal thresholds, indicative of an absence of hyperalgesia. These data confirm and extend previous findings from our lab and others demonstrating that CRF<sub>1</sub>R blockade reduces opioid withdrawal-induced hyperalgesia and attenuates heroin intake

post-escalation (Edwards et al., 2012; Greenwell et al., 2009; McNally and Akil, 2002). However, it is noteworthy that animals receiving chronic clonidine treatments appear to display alleviated hyperalgesia despite self-administering similar amounts of heroin as chronic vehicle-treated animals over the entire session (Figure 5-4E). These latter results suggest that a recruitment of adrenergic signaling occurs with extensive heroin exposure to drive hyperalgesia, although alleviation of opioid-induced hyperalgesia alone may be insufficient to block escalation of heroin intake. However, our evidence suggesting that clonidine tends to reduce intake in the first hour of heroin self-administration suggests that adrenergic system-mediated hyperalgesia may promote the initiation of opioid self-administration (Figure 5-4D).

These effects of clonidine also accord with a multi-system view of opioid addiction whereby altered central reinforcement mechanisms may continue to drive relapse to compulsive drug seeking despite an apparent alleviation of physical withdrawal symptoms (Schulteis and Koob, 1996; Self and Nestler, 1998). Moreover, as a pathological extension of hyperalgesia along the neuraxis, a greater interaction of supraspinal pain and reinforcement circuitry to produce pain-induced emotional dysregulation (termed hyperkatifeia) may be necessary to drive opioid intake escalation (Ji et al., 2007; Shurman et al., 2010). In this regard, our data suggest that blockade of CRF<sub>1</sub>R signaling would be a more effective and comprehensive therapeutic strategy aimed at this critical association of pain and motivational processes underlying opioid addiction (Weiss et al., 2001; Edwards and Koob, 2010).

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Chapter 5, in full, has been submitted to *Addiction Biology*. Park PE, Schlosburg JE, Vendruscolo LF, Schulteis G, Edwards S, Koob GF. The dissertation author was the primary investigator and primary author of this paper.

## **CHAPTER 6**

### **Conclusions**

The goal of this dissertation was to better characterize the negative affective states of anxiety-like behavior and hyperalgesia during withdrawal from heroin, and determine the effects of the CRF-NE brain stress system on the negative affective states and compulsive heroin intake. The general hypothesis is that increased anxiety- and pain-like behavior contribute to opioid dependence and are mediated by the activation of the brain stress system. We aimed to characterize anxiety- and pain-like responses during acute withdrawal from heroin administration and withdrawal from heroin self-administration. We also wanted to explore the neuropharmacological mechanisms for increased anxiety-like behavior and pain responsiveness during withdrawal from heroin. We attempted to determine the driving force in the feed-forward CRF-NE brain stress system to better understand its role in contributing to the negative affective states presented during heroin withdrawal, and the effects of CRF/NE stress system in heroin intake and escalation.

In Chapter 2, various validation studies were performed using the acoustic startle response. Yohimbine dose-dependently potentiated startle and the 1.25 mg/kg dose significantly potentiated startle over different days of testing with positive correlation. This showed that ASR can be used repeatedly as an anxiety-like behavior measure. Saline-treated animals did not show a change in startle amplitude over multiple days, and habituation was not observed in our studies (data not shown). The increase in startle from yohimbine was blocked by chlordiazepoxide, a benzodiazepine, as shown previously in literature (Davis et al., 1993). Intracerebroventricular (ICV) CRF has shown to potentiate startle

previously (Swerdlow et al., 1986) and in our laboratory in the present study (Fig. 2-4). The in vivo activity in ICV CRF was confirmed with testing locomotor activity where CRF-treated rats showed significantly higher overall locomotor activity compared to saline-treated rats (Fig. 2-5). Spontaneous withdrawal from both morphine and heroin showed increases in ASR and the same magnitude of potentiation was observed with naloxone administration, confirming that the startle potentiation was due to withdrawal. Through these studies, we were able to validate the acoustic startle response as a repeatable behavioral measure for anxiety-like responses and show that ICV CRF, yohimbine, or morphine withdrawal potentiates startle.

Based on these results, ASR was used to further explore the role of CRF and NE in heroin withdrawal-potentiated startle (Chapter 3). We observed that acute withdrawal from heroin (2mg/kg, SC) as well as withdrawal from day 1 of self-administration from heroin significantly potentiated startle. Clonidine ( $\alpha_2$  agonist; 10 $\mu$ g/kg, SC) and MPZP (CRF<sub>1</sub>R antagonist; 10 mg/kg, SC) blocked the heroin withdrawal-potentiated startle. Previous studies showed that clonidine blocked morphine withdrawal-potentiated startle (Harris & Gewirtz, 2004), but here we showed that heroin withdrawal-potentiated startle was blocked by a CRF<sub>1</sub>R antagonist (See summary in Table 6-1). In order to determine potential directionality in the CRF-NE brain stress system, we tested clonidine on CRF-potentiated startle, and MPZP on yohimbine-potentiated startle. Clonidine blocked CRF-potentiated startle but MPZP only partially attenuated but did not completely block yohimbine-potentiated startle. These results suggest that CRF

may drive NE in the CRF-NE feed forward brain stress system and that CRF<sub>1</sub> receptors and  $\alpha$ 2 receptors play an important role in the anxiety-like behavior observed during withdrawal from heroin (Figure 6-1; Park et al., 2013).

We then characterized pain-like behavior in the subsequent chapters. Similar to anxiety-like behavior, we hypothesized increased pain-like behavior during withdrawal, and recruitment of CRF signaling in mediating this behavior. Chapter 4 explores the development of mechanical hypersensitivity during withdrawal from ethanol vapor exposure, and heroin or cocaine self-administration. Rats made dependent on heroin or ethanol developed mechanical hypersensitivity compared to nondependent rats. However, the PWT for short- or long-access cocaine self-administering rats did not change. Furthermore, the mechanical hypersensitivity in heroin or ethanol dependent rats was reversed with a CRF<sub>1</sub>R antagonist MPZP, suggesting a recruitment of CRF-regulated nociceptive pathways associated with intake and dependence (Edwards et al., 2012). The developments here prompted us to further look into the effects of CRF<sub>1</sub> receptor antagonism in the acute withdrawal model.

Chapter 5 shows the development of hyperalgesia in rats undergoing acute withdrawal from chronic heroin injections. After 5 days of heroin (1.25 mg/kg, SC) injections, we observed the development of hyperalgesia and a downward shift of the PWT curve representing tolerance. In comparison, PWT of heroin self-administering rats were also tested. Similar to previous results (Edwards et al., 2012), we found that the PWT of self-administering rats continued to decrease only in LgA rats as they increase heroin intake, suggesting

that repeated heroin injections mimic the effects observed in heroin dependent rats that display escalated heroin self-administration. We then tested the hypothesis that CRF or NE had a role in the pain-like behavior observed during heroin withdrawal. Rats were given a series of heroin injections and once hyperalgesia developed, the CRF<sub>1</sub> receptor antagonist MPZP, or noradrenergic antagonists were tested during acute withdrawal. The CRF<sub>1</sub>R antagonist MPZP (20 mg/kg, SC) reversed the hyperalgesia. However, the NE antagonists did not significantly alleviate the hyperalgesia developed during acute withdrawal. Hyperalgesia was also reversed with chronic MPZP treatment given over 14 heroin self-administration sessions. Interestingly, chronic clonidine reversed hyperalgesia during withdrawal from self-administration while one administration of clonidine did not change PWT during acute withdrawal from heroin injections. These results indicate that CRF<sub>1</sub> receptors mediate pain-like behavior during acute withdrawal in the initial stages of dependence as well as withdrawal-induced heroin self-administration but the effects of clonidine require the development of dependence (See summary in Table 6-2).

Lastly, chronic treatment with MPZP prior to self-administration sessions blocked escalation of heroin intake while treatment with clonidine ( $\alpha$ -2 agonist) or vehicle did not block heroin escalation. Consistent with previous findings where CRF<sub>1</sub> receptor antagonist R121919 blocked established heroin escalation of intake (Greenwell et al., 2009), we observed a significant difference in heroin intake between vehicle and MPZP towards the end of the escalation period (treatment days 12, 13, 14). Chronic clonidine treatment did not have an effect on

heroin intake over the full 8-hour session. However, clonidine tended to reduce intake in the first hour of heroin self-administration, more or less at all time points, but did not blunt the slope of escalation. Here, alleviation of hyperalgesia was observed with chronic clonidine after escalation, but previous studies showed that clonidine did not reverse acute withdrawal induced hyperalgesia. This suggests opioid induced hyperalgesia alone may be insufficient to drive escalation of heroin intake. However, as dependence develops, hyperalgesia may involve an adrenergic component. These results indicate that CRF<sub>1</sub>R are involved in mediating anxiety- and pain-like behavior during withdrawal from heroin, as well as heroin self-administration. Noradrenergic systems may contribute to the negative emotional state as dependence evolves again arguing for a CRF- NE directional neuroadaptation.

In summary, we found that anxiety-like behavior increased during opioid withdrawal measured by the acoustic startle response (Chapters 2 & 3). Similarly, pain-like behavior increased during opioid withdrawal (Chapters 4 & 5). Increased mechanical sensitivity was observed in rats undergoing acute heroin withdrawal. Paw withdrawal thresholds decreased after chronic heroin injections, and the threshold curve showing the analgesic/hyperalgesic profile shifted downward (Figure 5-1). Throughout the escalation period of heroin self-administration, the paw withdrawal thresholds continued to decrease, representing the development of hyperalgesia parallel to the increase in heroin intake. Thus, acute systemic administration of opioids and extended access to



heroin self-administration reliably produced increased anxiety-like behavior and mechanical hypersensitivity.

Through pharmacological testing, we determined that CRF<sub>1</sub> receptors play an important role in the startle potentiation and hyperalgesia observed during opioid withdrawal, and CRF may be the driving force of the CRF-NE positive feedback loop. The CRF<sub>1</sub> receptor antagonist MPZP blocked the startle potentiation observed during acute withdrawal from heroin. Similarly, MPZP dose-dependently reversed the hyperalgesia during withdrawal from heroin self-administration. MPZP also reversed the hyperalgesia during acute withdrawal and in self-administering animals compared to vehicle. On the other hand, noradrenergic receptors seem to have variable effects on anxiety-like behavior and hyperalgesia. During acute withdrawal from heroin,  $\alpha$ 2 agonist clonidine blocked the startle potentiation but did not reverse the hyperalgesia. Chronic clonidine treatment given over the escalation period, however, reversed the hyperalgesia observed during withdrawal from heroin self-administration. The  $\alpha$ 1 antagonist prazosin or the  $\beta$  antagonist propranolol did not fully block the startle potentiation during acute heroin withdrawal and did not significantly reverse the hyperalgesia (Figure 6-2).

Both MPZP and clonidine were tested as potential prophylactic treatments prior to escalation of heroin self-administration and we found that MPZP, but not clonidine, showed a significant difference in heroin self-administration compared to vehicle on days 12, 13, and 14. MPZP-treated rats showed a relatively flat intake curve compared to clonidine- or vehicle-treated rats. Clonidine did not

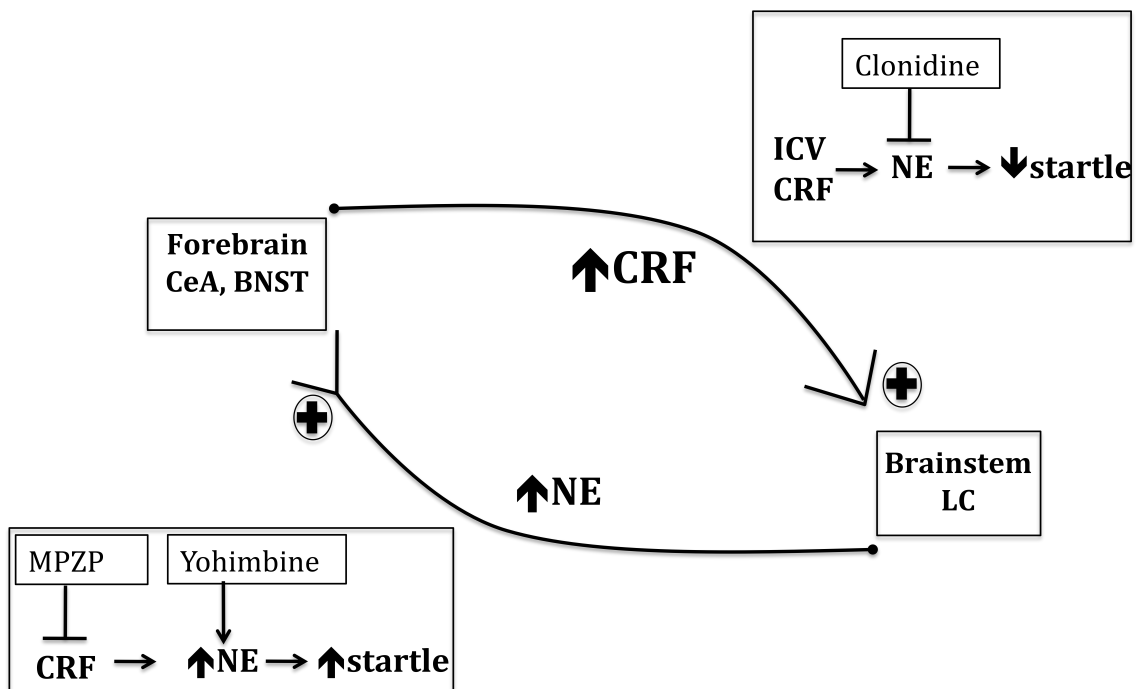
significantly lower heroin intake over the 8h session. However, we saw a trend in lower intake in the first hour of self-administration, suggesting a direct noradrenergic contribution to heroin self-administration independent of dependence induction (Park et al., submitted). In summary, CRF<sub>1</sub> receptor antagonism reversed both pain- and anxiety-like behavior observed during acute withdrawal from heroin, and blocked escalation of heroin intake. Noradrenergic antagonism showed differing effects in pain- or anxiety-like behavior, showing a dissociation between acute and chronic negative affective states.

Through the studies shown in this thesis, a few important discoveries were made in the field of addiction research (Figure 6-3). The role of CRF and NE were confirmed within the feed-forward brain stress system in mediating the anxiety-like behavior during withdrawal from heroin. CRF<sub>1</sub>R also seemed to play an important role in pain-like behavior observed during withdrawal but noradrenergic receptors did not seem to have the same effect as in anxiety-like behaviors. These results suggest the dissociation between pain and anxiety states during withdrawal that are usually hard to distinguish from one another. Furthermore, CRF<sub>1</sub> receptors mediated the escalation in heroin intake behavior while blocking NE altered heroin intake independent of escalation. Future studies utilizing brain region specific administration of CRF antagonists or NE antagonists would be the next step. This would confirm the location of action of CRF or NE, and a more specific circuitry for the CRF-NE stress system may be produced. Ultimately, the goal is to understand the circuitry of the brain stress system and its role in opioid addiction and develop pharmacological agents

altering the CRF-NE stress system as candidates for the treatment or prevention of opioid addiction.

**Table 6-1.** Summary of anxiety-like behavior using ASR.

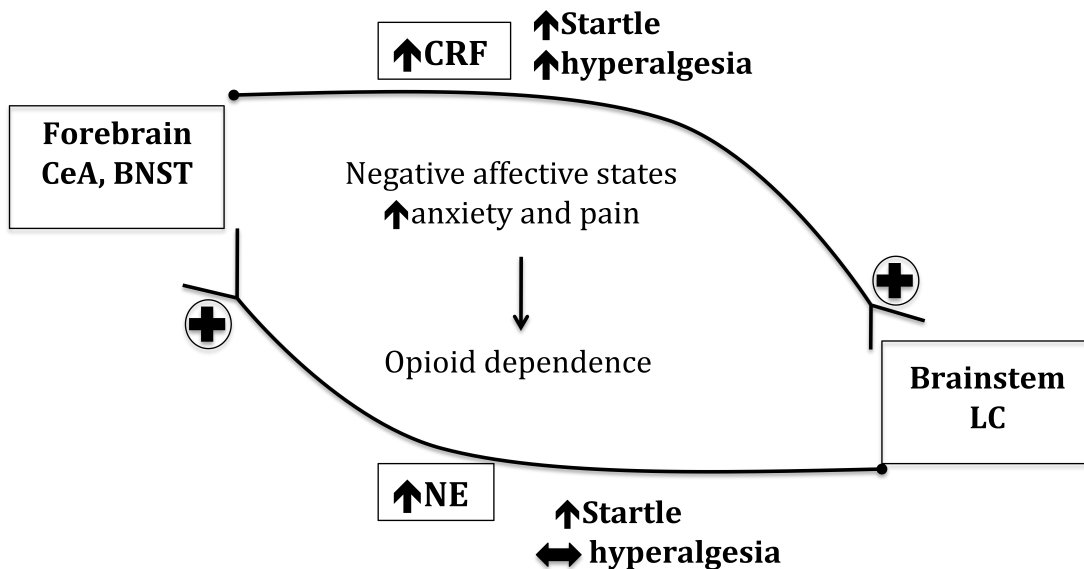
	<b>ASR</b>
Yohimbine	↑
ICV CRF	↑
Morphine withdrawal	↑
Heroin withdrawal	↑
CRF <sub>1</sub> R antagonism (MPZP)	+
	(Block withdrawal-potentiated startle)
α-2 agonism (clonidine)	+
	(Block withdrawal-potentiated startle)



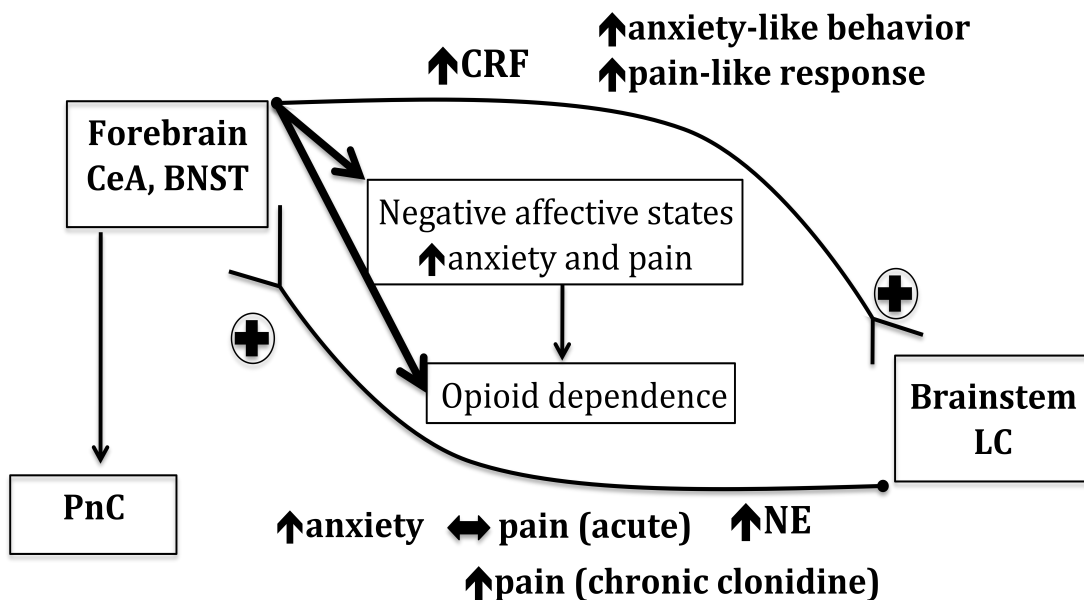
**Figure 6-1.** CRF may drive NE in the CRF-NE brain stress system. Blocking CRF did not fully block yohimbine-potentiated startle. However, clonidine was able to block CRF-potentiated startle. These results suggest that CRF may precede NE in the CRF-NE feed-forward loop and that CRF may be the driving force in the brain stress system, further activating NE release.

**Table 6-2.** Summary of pain-like behavior using mechanical sensitivity testing.

	<b>Acute Dependence</b>	<b>Self-administration</b>
Hyperalgesia	↑	↑
CRF <sub>1</sub> R antagonism (MPZP)	+	+
	(Reverses withdrawal-induced hyperalgesia)	(Reverses withdrawal-induced hyperalgesia)
NE blockade	No change	+
		(Chronic clonidine reverses hyperalgesia)



**Figure 6-2.** The effects of CRF and NE on startle and hyperalgesia. CRF plays a role in affecting both startle and acute withdrawal-induced hyperalgesia. NE affects ASR but does not reverse withdrawal-induced hyperalgesia in the acute dependence model. The CRF-NE brain stress system plays a role in displaying increased anxiety- and pain-like behavior, which ultimately influences opioid intake/dependence.



**Figure 6-3.** Summary of findings. CRF plays a role in anxiety- and pain-like behavior as well as opioid intake. NE seems to affect anxiety-like behavior but not acute heroin withdrawal-induced hyperalgesia. However, chronic clonidine reversed hyperalgesia during withdrawal from heroin self-administration. This CRF-NE stress system is part of a larger circuitry involved in mediating pain and anxiety-related behaviors during withdrawal. For example, CRF projections from the extended amygdala to the pontine reticular nucleus (PnC) modulate the enhancement of startle. Overall, this brain stress system is an integral part of the withdrawal/negative affect stage of the addiction cycle.



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