UNIVERSITY OF CALIFORNIA, SAN DIEGO

Attenuation of Brain Reward Thresholds during Acute Opioid Withdrawal by Antagonism of CRF Receptors in the Extended Amygdala

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Susanne Chang

Committee in charge:

Professor Gerhard Schulteis, Chair
Professor William Kristan, Co-Chair
Professor Randolph Hampton
Professor Susan Powell

2011

The thesis of Susanne Chang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

Co-Chair

_____

Chair

University of California, San Diego

2011

Table of Contents

List of Abbreviations

BNST	Bed Nucleus of the Stria Terminalis
CeA	Central Amygdala
CNS	Central Nervous System
CRF (-R1, -R2)	Corticotropin Releasing Factor (Type 1, Type 2 Receptor)
IC	Intracerebral
ICV	Intracerebroventricular
ICSS	Intracranial Self-Stimulation
MFB	Medial Forebrain Bundle
NAC	Nucleus Accumbens
SC	Subcutaneous

List of Figures and Tables

ABSTRACT OF THE THESIS

Attenuation of Brain Reward Thresholds during Acute Opioid Withdrawal by Antagonism of CRF Receptors in the Extended Amygdala

by

Susanne Chang

Master of Science in Biology

University of California, San Diego, 2011

Professor Gerhard Schulteis, Chair
Professor William Kristan, Co-Chair

Increased release of the stress neurotransmitter corticotropin-releasing factor (CRF) in regions of the extended amygdala has been shown to mediate drug withdrawal behavior. These symptoms of withdrawal have been characterized by dysphoria-like signs that can be measured by assessing levels of brain reward deficits using intracranial self stimulation (ICSS). In an initial study, the dose-response curve for naloxone-precipitated elevations in brain reward thresholds following an acute or repeated (4x at daily intervals) daily injections of 10 mg/kg

of morphine was determined. Using a discrete-trial brain stimulation reward procedure, male Wistar rats implanted with stimulating electrodes aimed at the medial forebrain bundle received either repeat- or acute- treatment of morphine (10 mg/kg) 4 hr prior to one of several doses of naloxone (0.01, 0.033, 0.33. 1.0 mg/kg); naloxone was administered just prior to a test session. Naloxone dose-dependently increased thresholds after acute and repeat morphine, and its potency was greater after repeated morphine treatments. Selecting an optimal dose of naloxone (1.0 mg/kg) under acute morphine pretreatment conditions, a second study examined the effects of bilateral infusion of the CRF-R1 antagonist, antalarmin (1.0, 3.3 nmol), into the CeA and NAC shell. Results suggest that 4 h after an acute morphine (10 mg/kg) pretreatment, the highest dose of antalarmin tested (3.3 nmol) in both brain regions significantly attenuated naloxone-induced elevations in reward thresholds. The data indicate that preventing endogenous CRF in the CeA and the shell of the NAC from binding to CRF-R1 receptors can significantly blunt the brain reward deficits that result from precipitated opioid withdrawal.

1. Introduction

Recreational drug abuse and addiction significantly affects society in terms of human suffering, legislative and bureaucratic burden, crime, disease, and healthcare costs. Opioid analgesic abuse and overdoses have shown alarming growth between the years1999 and 2008 (Paulozzi et al., 2011). The typical recreational drug user often initiates drug use to experience the pleasurable (rewarding) effects of a drug and/or for self-medication of pre-existing, emotional disorders (Mills et al., 2006). For example, among patients prescribed opioids for non-cancer pain, those who are also diagnosed with anxiety, depression, or post-traumatic stress disorder (PTSD) show significant co-morbidity risk to chronic opioid use (Sullivan et al., 2005). As use continues and escalates into a state of dependence, continued use may furthermore be motivated by self-medication of the aversive withdrawal symptoms that additionally mimic or exacerbate symptoms of these stress disorders (Newport and Nemeroff, 2000). The state of drug addiction includes a state of compulsive use, drug-seeking behavior, and negative affective states (e.g., dysphoria, anxiety) during cessation of drug use (Koob and Le Moal, 1997). The combination of negative withdrawal symptoms and the positive emotional effects resulting from drug intake produce a highly motivating drive to compulsive drug use characteristic of addiction.

Not all drug use leads to addictive patterns of behavior, so understanding the factors contributing to the transition from casual drug use to loss of control, and compulsive use is necessary to determine the underlying factors in vulnerable individuals. Identifying the early symptoms of acute drug use may provide the basis of understanding the differences in susceptibility between individuals due to neuroadaptive responses to opioids and other drugs of abuse. In users prone to more rapid addiction, acute withdrawal symptoms from individual bouts of drug intake may motivate the user to self-medicate in order to minimize negative emotional signs during periods of (Schulteis, 2010a). Acute dependence has been defined as the “state in which abstinence can be demonstrated or precipitated following either an acute dose or a short-term infusion of [drugs]” (Martin and Eades, 1964). Similar affective or emotional signs of withdrawal are observed following acute or chronic opioid treatment or use in humans (Azorlosa et al., 1994) and animals (Azar et al., 2003; Liu and Schulteis, 2004; Parker and Joshi, 1998), including anxiety, dysphoria, and irritability. In addition to opioids, other drugs of abuse (e.g., alcohol, benzodiazepines and barbiturates, amphetamines) also can engender acute drug dependence that is characterized by negative emotional signs of withdrawal (Schulteis, 2010a—the Acute Dependence Chapter).

One of the more prominent emotional symptoms of withdrawal in human addicts is “dysphoria”, characterized as "a state of feeling unwell or unhappy [and] opposite to euphoria (Schulteis, 2010b)." In animal models, dysphoria-like

effects can be measured using intracranial self-stimulation (ICSS) reward thresholds as a sensitive index (Schulteis, 2010b). A decrease in brain stimulation reward (BSR) threshold is observed during the euphoria-like states of initial drug exposure to drugs of abuse such as opioids, ethanol, cocaine and amphetamines (reviewed in Schulteis, 2010b). These drugs are related in their abuse potential and appear to be mediated by the reward circuitry in the “extended amygdala” which suggests a euphoria-like effect that acts through a common neural circuitry (Kornetsky and Bain, 1990).

Alternately, during withdrawal from habit-forming drugs, an increase in the brain stimulation reward is required to elicit reliable self-stimulation behavior (Johnson et al., 2008; Lin et al., 1999; Markou and Koob, 1991; Schulteis et al., 1995; 1994). The neural circuitry mediating the pleasurable effects of abused drugs also mediate negative emotional states such as dysphoria observed during withdrawal once the individual is dependent. This "extended amygdala" circuit is centered on a collection of structures that includes portions of the midbrain, diencephalon and basal forebrain that share similar morphology, immunohistochemistry and connectivity (Alheid and Heimer, 1988), and has been hypothesized to form the neural pathway for reward. Particularly relevant to drug reward and dependence are the following elements of extended amygdala components: bed nucleus of the stria terminalis (BNST), central amygdala (CeA) and shell of the nucleus accumbens (NAC) (Heimer and Alheid, 1991). These regions showed high levels of *c-Fos* immunopositive neuronal cell bodies upon precipitated withdrawal from morphine dependence (Veinante et al., 2003). The

extended amygdala receives numerous afferents from limbic structures such as the ventral tegmental area (VTA), basolateral amygdala and hippocampus and sends efferents to the medial part of the ventral pallidum and large projections to the lateral hypothalamus (Alheid et al., 1995). Thus, the extended amygdala provides a connection for the basal forebrain to the lateral hypothalamus via the medial forebrain bundle hypothesized to be the brain's reward system (Koob, 2006) by providing a coordinated system for motor and autonomic responses. The extended amygdala may be a key substrate that undergoes rapid neuroadaptation in response to acute drug exposure, as well as rapid escalation of this adaptive response with repeated intermittent drug exposure.

Of particular interest in the extended amygdala is the increase in activity of the corticotropin-releasing factor (CRF) stress system in response to physiological and psychological stressors, like withdrawal from drugs of abuse (Funk et al., 2006; Olive et al., 2002; Richter and Weiss, 1999). CRF is a 41 amino acid peptide found in the CNS in the paraventricular nucleus of the hypothalamus (PVN) as well as extrahypothalamic regions such as the limbic system known to mediate behavioral response to stress: CeA, BNST, and hindbrain regions (Bale and Vale, 2004; Heinrichs et al., 1995b; Sarnyai et al., 2001). The actions of CRF are mediated by two major types of G protein-coupled receptors that mostly have distinct regional distribution throughout the central nervous systems and periphery: CRF receptor 1 (CRF-R1) and CRF receptor 2 (CRF-R2). CRF-R1 is distributed throughout the cerebral cortex and cerebellum with receptors in the basolateral amygdala (BLA), CeA, medial amygdala (MeA),

medial septum, BNST, NAC, and VTA (Potter et al., 1994; Van Pett et al., 2000). CRF-R2 is evident in the BNST, ventromedial hypothalamic nucleus and the choroids plexus in the CNS (Chalmers et al., 1995).

Several studies have shown that an intracerebroventricular (ICV) infusion of CRF produces behaviors in rodents indicative of stress, such as a decrease in exploration behavior, increased acoustic startle reflex, and decreased exploration of the open arms in the elevated plus maze test (Dunn and Berridge, 1990). CRF appears to elicit an overall stimulatory response to stress through the activation of CRF-R1, while studies suggest that CRF-R2 binding dampens and regulates the stress response (Bale and Vale, 2004). CRF-R1 antagonists successfully reverse stress-inducing responses, including the anxiety and aversive states of drug withdrawal (Zorrilla and Koob, 2004). In addition, all major regions in the extended amygdala thought to be involved with drug addiction express CRF-R1 receptors, but not all express CRF-R2 (Van Pett et al., 2000). Therefore, the majority of studies have focused on the administration of CRF-R1 antagonists as the primary mechanism to reverse the behavioral/emotional consequences of withdrawal from drug dependence. Recruitment of the CRF system has been hypothesized to be involved in mediating the pathway to drug dependence (Koob, 2008).

Elevations of CRF levels are seen during withdrawal from drugs of abuse, which activate the brain stress system. CRF-R1 antagonists have successfully prevented withdrawal-induced elevations in brain reward thresholds of nicotine dependent rats (Bruijnzeel et al., 2009), alcohol dependent rats (Bruijnzeel et al.,

2010), and recently, the effects of a systemically-administered CRF-R1 antagonist has also been shown to attenuate reward deficits during opioid withdrawal (Lonergan et al., submitted).

Morphine is well characterized in its course of acute and chronic opioid dependence. The extended amygdala is an important location of μ-opioid receptors mediating reward and antagonist-precipitated withdrawal from acute and repeated morphine exposure (Criner et al., 2007). Morphine has a high affinity to μ-opioid receptors, which is the primary activation site inducing acute opioid dependence (Schulteis, 2010a). Availability of reliable competitive antagonists such as naloxone and naltrexone is ideal in order to facilitate the precipitation of withdrawal in studies of opioid dependence (Criner et al., 2007) Intracerebral application of the opioid antagonist methylnaloxonium to the NAC, CeA and BNST to acute and chronically opioid-dependent rodents selectively elicits aversive motivational signs of withdrawal as measured by suppression of operant responding for food reward (Criner et al., 2007). Negative emotional signs of withdrawal from acute opioid dependence has been measured by ICSS (Easterling et al., 2000), conditioned place aversion (CPA) (Heinrichs et al., 1995b) and anxiety-like behavior (Zhang et al., 2008).

The initial portion of the study will focus on determining the dose-response effect of the morphine antagonist, naloxone, after acute and repeated-intermittent morphine 10 mg/kg administration. While naloxone-precipitated withdrawal from morphine 5.6 mg/kg has successfully produced reliable effects in previous studies using ICSS and food reward measures of withdrawal (Criner et al., 2007;

Liu and Schulteis, 2004), recent studies testing anxiety-like behavior during withdrawal from acute and repeated-intermittent morphine failed to show naloxone dose-dependency at 5.6 mg/kg of morphine (Zhang et al., 2008). However, dose-dependent sensitivity to naloxone-precipitated anxiety-like behavior was observed when using morphine 10 mg/kg. Therefore to allow a more direct comparison of brain substrates across anxiety and reward-deficit sings of withdrawal, the first experiment determined the naloxone dose-effect function under a standardized 10 mg/kg morphine dose.

Selecting an optimal dose of naloxone from this initial experiment, subsequent experiments examined whether CRF in either the CeA or NAC shell, or both, might contribute to elevated reward thresholds during withdrawal from acute exposure to morphine. Preliminary data indicates that systemic administration of a CRF-R1 antagonist (MPZP) attenuated naloxone-precipitated ICSS reward threshold elevations after repeated injections of 5.6 mg/kg morphine (Lonergan et al., submitted). An additional purpose of this study was therefore examining the effects of withdrawal from an acute treatment of morphine, to provide insight into whether changes in CRF systems contribute to the neural adaptations seen after initial drug exposure. The CRF system in the CeA and the NAC shell have been strongly implicated in mediating drug withdrawal responses (Heinrichs et al., 1995b; Swiergiel et al., 1993; Veinante et al., 2003), so these regions were targeted in the present study.

The selective CRF receptor-1 antagonist, antalarmin, has successfully attenuated aversive signs of morphine withdrawal (Stinus et al., 2005). In the

present study, antalarmin was infused into discrete components of the extended amygdala (CeA and NAC) via surgically implanted guide cannulae to examine the extent to which these brain regions showed antalarmin-induced reversal of dysphoria-like effects during withdrawal from acute morphine treatment, as measured in the ICSS model. The overarching goal of this project was to gain an increased understanding of the neuroadaptive circuit in the extended amygdala during the early onset of drug dependence and provide a possible therapeutic target for drug addiction treatment.

2. Materials and Methods

2.1 Animals

Male Wistar rats from Harlan Sprague Dawley (Livermore, CA) weighed 200-250 g upon arrival. Rats had *ad libitum* access to food and water, except during ICSS sessions, and were pair-housed. Rats were acclimated to their housing conditions, including a 12-hour light/dark cycle (lights on at 6:00 am), for at least one week before handling and surgery. Testing occurred between the hours of 8:00 am and 5:00 pm from Monday through Friday. All procedures and facilities used in the study were approved by the Institutional Animal Care and Use Committee (IACUC) of the VA San Diego Healthcare System, which is fully-accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.2 Surgical and Injection procedure

Rats were anesthetized with 2-5% isoflurane in oxygen and secured in a Kopf stereotaxic instrument with the incisor bar set at -3.3 mm below the interaural line for perpendicular implantations relative to the surface of the skull. Instruments were heat sterilized in glass beads before the procedure, and cannulae were pre-sterilized in Betadine and rinsed with 70% alcohol. A unilateral, bipolar electrode (Plastics One) was implanted in the medial forebrain bundle, alternating between the left and right side of the brain for a given rat, with the coordinates at: AP -2.8 mm and ML +1.7 or -1.7 mm using bregma as a reference point and DV -7.9 mm was measured from the dura layer. The electrode probes were separated by 1 mm, and extended 10 mm from the plastic cap.

Animals that required cannulation were implanted with additional stainless steel, bilateral guide cannulae (SmallParts, 23 gauge, 12.5 mm) through burr holes in the skull at sites directly above the corresponding regions of interest, and all coordinates were measured from the skull surface, at bregma. CeA coordinates were: AP -2.2 mm, ML ±4.2 mm, DV -8.4 mm and NAC coordinates are: AP +1.6 mm, ML ±1.3 mm, DV -7.9 mm. The atlas of Paxinos and Watson (2007) provided the coordinates for cannula placement. Separate groups of rats used as diffusion controls were implanted with cannulae 1 mm short of the target depth for each site. The depth of the guide cannulae terminated 2.5 mm above the desired location to avoid tissue damage to the region of interest; cannula-length stylets (SmallParts, 30 gauge) were inserted to maintain the patency of

the cannula until infusion. Because of the proximity between the electrode and the CeA guide cannulae, the electrode was extended to 16 mm in total length and bent at two right angles to accommodate placement of the screw mount for the electrode away from the CeA guide cannulae. Dental cement poured around the guide cannulae and electrodes, and over six anchor screws helped secure the implanted cannulae and electrodes to the skull. At the end of the surgical procedure, 0.2 mL of 1% Bupivicaine was topically applied around the base of the cap, followed by antibiotic ointment. Rats were allowed one week to recover before operant training.

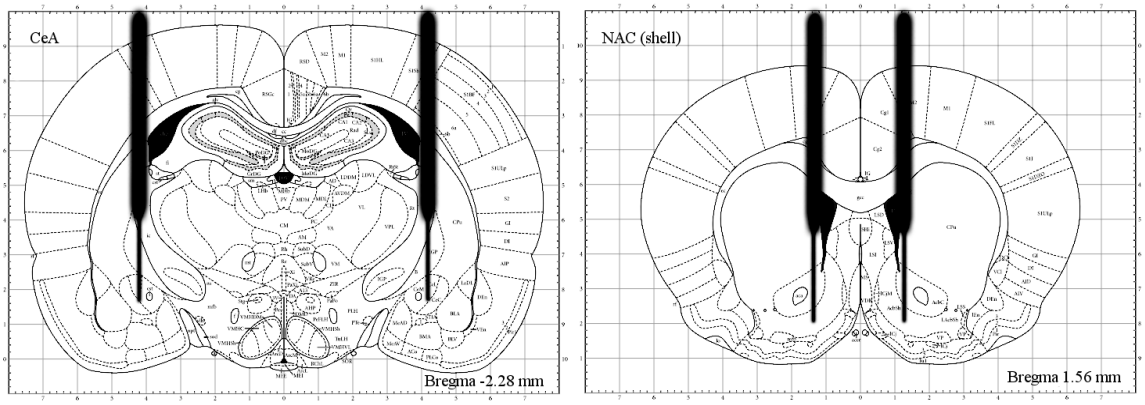


Figure 1. Schematic representation of the guide cannulae (broad bars) and injector cannulae (thin bars) targets for the CeA and the NAC (shell) regions. The CeA image represents a coronal brain section -2.28 mm relative to bregma. The NAC shows a coronal slice at 1.56 mm relative to bregma. The images are adapted from the atlas of Paxinos and Watson (2007).

To perform the intracerebral (IC) injections, dummy stylets were removed and replaced by injector cannulae (30 gauge, 15 mm long) which extended 2.5 mm beyond the end of the guide cannula. Polyethylene tubing connected the injector cannulae to a microsyringe, which was driven by a Razel microsyringe

pump that delivered 0.5 µl of the drug solution through bilateral injectors over a 60 sec infusion period. After infusion, the injectors were left in place for at least 90 sec to maximize diffusion and minimize efflux up the cannula tract. Stylets were immediately re-inserted into the guide cannulae after infusion.

2.3 Drugs

Morphine sulfate (Sigma-Aldrich, MO) and Naloxone HCl (Sigma-Aldrich) salts were dissolved in 0.9% physiological saline, and were both injected subcutaneously (SC) in a volume of 0.1 ml/100 g of body weight. Drug doses were expressed as the salt. Antalarmin hydrochloride (Sigma-Aldrich) was prepared immediately prior to use according to the procedure described by Henry et al. (2006) and dissolved in a solution of: 85% sterile saline, 10% Cremaphor (Sigma-Aldrich) and 5% ethanol. Antalarmin doses were calculated as total dose infused bi-laterally, with half of the dose infused into each hemisphere in a volume of 0.5 µl per side. Control injections consisted of the appropriate vehicle solution used to solubilize the drug.

2.4 Verification of surgical cannula placements

After completion of testing, injector-length stylets were dropped into the cannula opening and cemented in place. After 5 days, rats were overdosed with an IP injection of 0.4 ml of Euthasol, then transcardially perfused with 10%

buffered formalin. The brains were collected and stored in 10% formalin fixative for 24 hours. The solution was then changed over to a 30% sucrose solution in PBS until the brain sank to the bottom of the collection vial. The brains were then wiped dry and frozen in Optimum Cutting Temperature, then, coronally sectioned into 50 µm slices using a cryostat. Slides were dipped in a decreasing sequence of ethanol solutions, of 95%, 75%, 50% and then deionized water. They were subsequently dipped into a cresyl violet staining solution before being dehydrated again in increasing concentrations of ethanol, followed by a quick dip in deionized water. Sections were examined under a light microscope and the brain atlas *The Rat Brain in Stereotaxic Coordinates* by Paxinos and Watson (2007) was used as a guide. The terminal location of the injector stylet tip was recorded and mapped onto the appropriate section and location while blinded to the results of behavioral testing. Figure 2 illustrates the termination points of cannula with correct placements; the Figure is representative of the full range of cannula placements in all groups, although only certain groups are plotted in order to enhance clarity. The diffusion control histology is mapped to the right and shows the distinction between infusion locations. The NAC diffusion control site also served as a separate examination of the core region of the NAC which sits dorsally to the site of NAC shell injection. Only animals that clearly hit the mark were included into the study and all data from subjects with injector placements outside of the target regions were discarded.

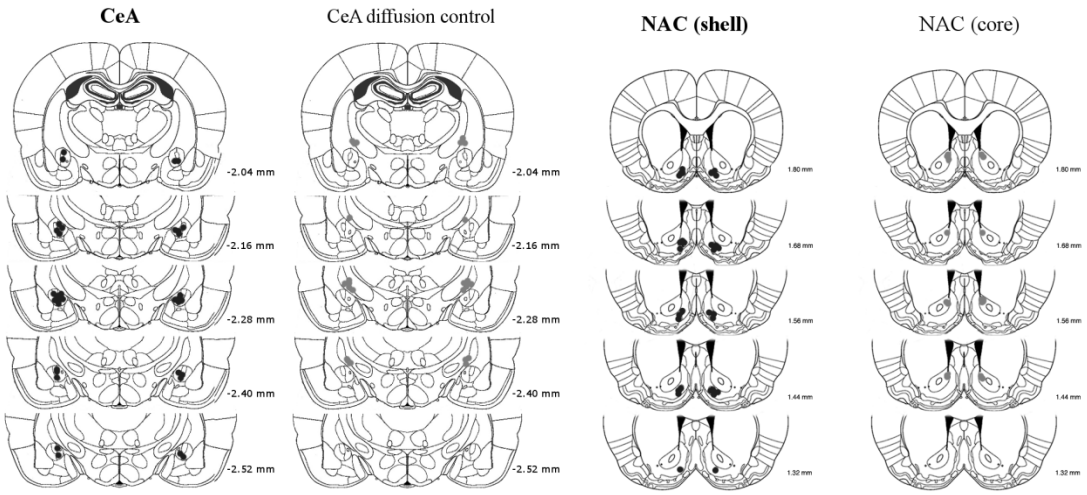


Figure 2. Injector cannula tip termination locations for the two highest doses of Antalarmin tested in the CeA and the NAC. Also included are the corresponding diffusion control sites positioned 1 mm above the target depth. Numbers on the side represent the AP distance from bregma. Images adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 2007).

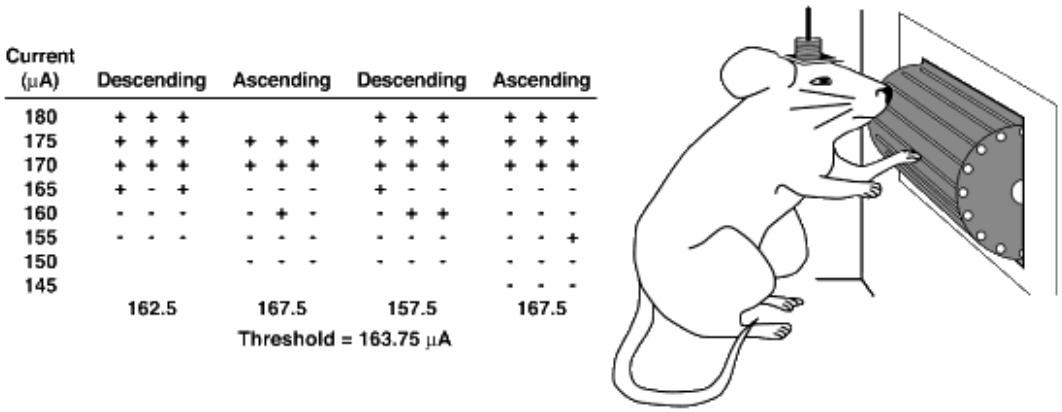
2.5 Discrete-Trial Brain stimulation reward procedure

The discrete-trial technique of intracranial self-stimulation (ICSS), using the psychophysical method of limits to determine thresholds, as pioneered by Kornetsky and Esposito (1979), characterizes drug-induced euphoria-like or the dysphoria-like signs that is experienced during drug use as alterations in reward thresholds. An electrode aimed at the medial forebrain bundle (MFB), provides brain stimulation, and an animal's willingness to receive a response-contingent electrical stimulation serves as a sensitive index of rewarding properties of the self-stimulation (Schulteis, 2010b). Alternate brain stimulation reward techniques measure the response rate at a particular current, stimulation frequency, and duration of stimulation with some success. However, under drug influences,

subjects often undergo behavioral deficits or sedative effects that are nonspecific to the rewarding effects of the drug and compete with high levels of operant that are required in these paradigms. Discrete-trial ICSS requires few total responses and reliably measures reward thresholds with minimal confound by non-specific motoric effects of drugs (Markou and Koob, 1992).

Rats were trained, without drugs, to respond to an intracranial self-stimulation (ICSS) current of 100 Hz sinusoidal waves with train duration of 250 ms. Upon first exposure to the apparatus, the animal received a response-contingent stimulus with every ¼ turn of the wheel until the animal achieved around 100 successful rewards within a two minute window at a given current. The animal then moved on to a more complex session in which a ¼ wheel turn was reinforced by electrical stimulation, but with increasing delays between successive reward opportunities of 1, 5, 10, and 15 sec (i.e. responses during the 1-15 sec “timeout” interval did not result in electrical stimulation, thereby training the rat to minimize response rates). The rat was transitioned to the next step when a 40% reward success rate was achieved at a given interval. Upon completion of the second part of the training, the subject graduated to the final stage of training in which reward thresholds could be determined. In this phase of training, each trial started with a noncontingent electrical stimulus of a given current intensity delivered without requiring a response from the animal. The rat then had 7.5 seconds to respond by completing a ¼ rotation of the wheel. If successful, a reward was given that was identical in all parameters to the

previous noncontingent stimulus (positive response). If no response occurred within the 7.5 sec window after the noncontingent stimulus, then the trial was considered a negative response. The end of each trial was interspersed with an inter-trial interval ranging from 7.5-12.5 sec and averaged 10 sec. Any response during the inter-trial interval resulted in ~10-sec delay before the start of the next trial. Stimulus intensities varied and were presented in two series of alternating ascending and descending trials with a step size of 5 µA. The threshold for each series was the midpoint between the current intensity level at which the animal provides at least two positive responses (out of three presentations of a given current intensity) and the level at which fewer than two positive responses occurred over two consecutive intensities. As seen in Figure 3, the mean of the four series served as the estimated threshold for a given session.



Current (µA)	Descending	Ascending	Descending	Ascending
180	+ + +		+ + +	+ + +
175	+ + +	+ + +	+ + +	+ + +
170	+ + +	+ + +	+ + +	+ + +
165	+ - +	- - -	+ - -	- - -
160	- - -	- + -	- + +	- - -
155	- - -	- - -	- - -	- - +
150		- - -	- - -	- - -
145				- - -
	162.5	167.5	157.5	167.5

Threshold = 163.75 µA

Figure 3. Discrete trial ICSS procedure. The table represents a hypothetical model depicting how the threshold is established over a given session. Image adapted from Neurobiology of Addiction (Koob and Le Moal, 2006)

2.6 Experimental Design

2.6.1 Experiment 1: Naloxone Dose Response Determination

Morphine dose and interval between morphine administration and precipitation were adapted from previous work to determine optimal conditions and delay for *acute* opioid responses (Schulteis et al., 1997; Easterling et al., 2000; Liu and Schulteis, 2004). Rats were trained twice daily (an AM and a PM session separated by 4 hr) in the operant ICSS procedure for approximately a 3-week period until a stable baseline was reached with <15% variation in threshold over five consecutive days.

Baseline Phase: Over the 4-day baseline period, rats received a SC injection of saline after the AM session. Four hours after the first injection, rats received another SC injection of saline right before their PM session to habituate them to injection procedures.

Testing Phase: Once stable baseline thresholds for both AM and PM sessions were established, the 4-day testing phase was initiated. Animals were counterbalanced across testing groups according to baseline thresholds. Table 1 lists all combinations of treatments administered in this experiment. On days 1 to 3, only a single AM ICSS session was given, followed by a SC injection of either morphine (10 mg/kg) or saline, depending on the assigned treatment group (Repeat Morphine received morphine days 1-3, Acute Morphine groups received saline days 1-3; subjects did not run in a PM ICSS session on days 1-3 to

avoid development of conditioned withdrawal responses to the ICSS testing chambers. On day 4, rats again ran in an AM session of ICSS, which was followed by a SC injection of morphine for both Acute and Repeat Morphine conditions. Four hours after the morphine injection, either vehicle or a dose of naloxone was administered, ranging from 0.01 mg/kg to 1.0 mg/kg, preceding the PM ICSS run. Thresholds from the testing days were compared to the corresponding AM or PM thresholds established during the baseline period.

2.6.2 Experiment 2: CRF- R1 Antagonist Effects in CeA and NAC

The procedures and timing for testing and SC injections of vehicle, morphine, and/or naloxone were similar to those in the dose-response study. However, during the pre-treatment phase, 3 hour and 45 minutes after the initial injection, rats received a “mock” IC infusion where they were gently restrained while habituated to removal and re-insertion of the stylets; the rats were held while the infusion pump was turned on for about two minutes to also habituate them to the sounds of the pump and to being held during infusion. Rats were then placed back in their box until they received their second SC injection 15 minutes after the mock infusion, with the second ICSS session started within 5 min of this second SC injection.

The conditions employed during the testing phase, seen in Table 2, included only Morphine-Naïve (vehicle in place of morphine) or Acute

Morphine conditions, and vehicle or the 1.0 mg/kg dose of naloxone; this dose was selected from Experiment 1 as producing the highest threshold elevation with minimal inter-subject variability. On days 1 to 3, a single AM ICSS session was followed by a SC injection of saline. On day 4 of the testing phase, a SC injection of morphine (10 mg/kg SC) was given after the first ICSS session. Different groups were tested with either vehicle or one of the several doses of antalarmin (1.0, or 3.3 nmol, total dose) infused IC 3 hr 45 min later, followed by vehicle or naloxone (1.0 mg/kg) injected SC at 4 hr post-morphine. A second ICSS session was run within 5 minutes of the last SC injection. All groups had ICSS electrode implants, but IC implants in only one of the two brain regions of interest. Therefore, separate groups were tested with antalarmin infused into either CeA or NAC shell.

2.7 Statistical analysis

Data collected for testing trials were expressed as a percent of the threshold obtained during the baseline period of the respective AM or PM sessions for each rat. Results were analyzed through one-way or two-way analysis of variance (ANOVAs), as appropriate. *T*he level of statistical significance was held constant at $p < 0.05$, with Bonferroni corrections applied when multiple comparisons of two means followed overall significant outcomes in the ANOVA. Data were analyzed with JMP 9.0 for Macintosh (SAS, Cary NC).

Table 1. ICSS Dose Response Schedule

Treatment group	Baseline Days		Testing days 1-3		Testing day 4	
	AM post-run *(t=0)*	PM pre-run *(t=4)*	AM post-run *(t=0)*	PM pre-run *(t=4)*	AM post-run *(t=0)*	PM pre-run *(t=4)*
Vehicle-Vehicle	Vehicle	Vehicle	Vehicle	-	Vehicle	Vehicle
Acute Morphine-Vehicle	Vehicle	Vehicle	Vehicle	-	Morphine	Vehicle
Acute Morphine-Naloxone						
Naloxone 0.01	Vehicle	Vehicle	Vehicle	-	Morphine	Naloxone 0.01
Naloxone 0.033	Vehicle	Vehicle	Vehicle	-	Morphine	Naloxone 0.033
Naloxone 0.33	Vehicle	Vehicle	Vehicle	-	Morphine	Naloxone 0.33
Naloxone 1.0	Vehicle	Vehicle	Vehicle	-	Morphine	Naloxone 1.0
Repeat Morphine-Vehicle	Vehicle	Vehicle	Morphine	-	Morphine	Vehicle
Repeat Morphine-Naloxone						
Naloxone 0.01	Vehicle	Vehicle	Morphine	-	Morphine	Naloxone 0.01
Naloxone 0.033	Vehicle	Vehicle	Morphine	-	Morphine	Naloxone 0.033
Naloxone 0.33	Vehicle	Vehicle	Morphine	-	Morphine	Naloxone 0.33

Dash represents no treatment
[a] Naloxone doses represented in mg/kg

Table 2. ICSS Antalarmin Dose Response Schedule

Treatment group	Baseline Days			Testing days 1-3			Testing day 4		
	AM post-run	PM infusion	PM pre-run	AM post-run	PM infusion	PM pre-run	AM post-run	PM infusion	PM pre-run
	(t=0)	*(t=3h 45m)*	*(t=4)*	*(t=0)*	*(t=3h 45m)*	*(t=4)*	*(t=0)*	*(t=3h 45m)*	*(t=4)*
Veh-Veh-Veh	vehicle	mock	vehicle	vehicle	-	-	Vehicle	Vehicle	Vehicle
Veh-Veh-Nal	vehicle	mock	vehicle	vehicle	-	-	Vehicle	Vehicle	Vehicle
Veh-Ant 3.3-Nal	vehicle	mock	vehicle	vehicle	-	-	Vehicle	Ant 3.3[a]	Naloxone[b]
Mor-Veh-Veh	vehicle	mock	vehicle	vehicle	-	-	Morphine[c]	Vehicle	Vehicle
Mor-Veh-Nal	vehicle	mock	vehicle	vehicle	-	-	Morphine	Vehicle	Naloxone
Mor-Ant-Nal									
Mor-Ant 1.0-Nal	vehicle	mock	vehicle	vehicle	-	-	Morphine	Ant 1.0	Naloxone
Mor-Ant 3.3-Nal	vehicle	mock	vehicle	vehicle	-	-	Morphine	Ant 3.3	Naloxone
Mor-Ant 3.3-Nal[d]	vehicle	mock	vehicle	vehicle	-	-	Morphine	Ant 3.3	Naloxone

Dash represents no treatment
[a] Infused drug measured in total nmol IC
[b] Naloxone administered SC in 1.0 mg/kg
[c] Morphine administered SC in 10 mg/kg
[d] diffusion control

3. Results

3.1 Experiment 1: Naloxone Dose Response Determination

Prior to administration of naloxone on day 4, all groups in the Morphine-Naïve, Acute Morphine, and Repeat Morphine conditions were treated equally; therefore absolute AM and PM baseline reward thresholds (Figure 4) were analyzed with respect to Morphine test condition, but not with respect to naloxone condition. Among all test conditions, there were no significant differences in baseline threshold values as measured by the main effect of treatment condition [$F(2,124)=.34$, $P=.71$] and the treatment x time interaction [$F(2,124)=1.165$, $P=.315$] in a mixed-design two-factor ANOVA with treatment condition as a between-subjects factor and test time (AM vs. PM session) as a repeated measure. However, the main effect of test time revealed a significant difference between the AM and PM baselines (Figure 4), with slightly higher threshold values during the PM runs [$F(1,124)=22.74$, $P<.0001$]. Accordingly, test data were always compared against the corresponding AM or PM baseline threshold.

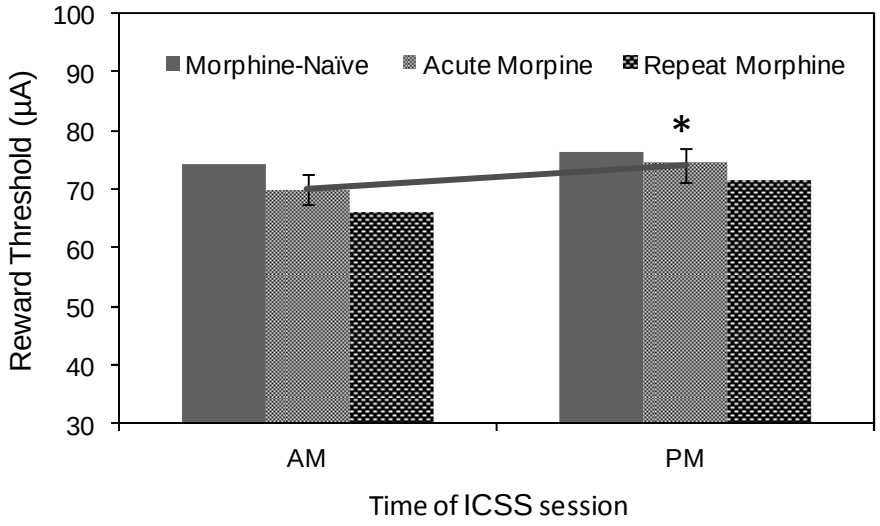


Figure 4. Average threshold values under the various morphine conditions for the AM and PM ICSS sessions. The line represents the overall main shift in threshold (±S.E.M.) over time. (*P*<.001; significance of AM threshold vs. PM threshold).

On the first testing run (AM day 1), prior to any morphine treatment, as seen in Figure 5, no difference in % brain reward threshold (BRT) was observed between rats in the different morphine conditions [$F(2,124)=.48$, $P=.62$]. Therefore, any deviations in AM thresholds after day 1 can be attributed to the different conditions imposed on the rats following the initial test. In a two-factor mixed design ANOVA with morphine condition (Acute or Repeat) as a between-subjects factor and test day as a repeated measure, there was significant main effect of morphine condition [$F(1,125)=38.12$, $P<.001$], as well as a main effect of test day [$F(2,124)=18.95$, $P<.001$] and a significant morphine condition x test day interaction [$F(3,123=14.69)$, $P<.001$]. The interaction was due to a significant rise in the thresholds of rats in the Repeat Morphine but not Acute Morphine conditions on Days 2, 3 and 4 of testing (each AM threshold on Days 2, 3 and 4

is 23 hr after a preceding morphine injection in the Repeat Morphine group). Post-hoc comparisons revealed that the increase in AM session threshold in the Repeat Morphine group on Days 2, 3 and 4 was significant relative to threshold on Day 1. A similar finding was seen by Liu and Schulteis (2004), using a 5.6 mg/kg dose of morphine, although in that study significant increases in threshold were not observed until Day 3 (23.5 h after the second morphine treatment). The results are consistent with the emergence of a state of spontaneous (not precipitated) withdrawal that can be seen 23 hr after a prior dose of morphine.

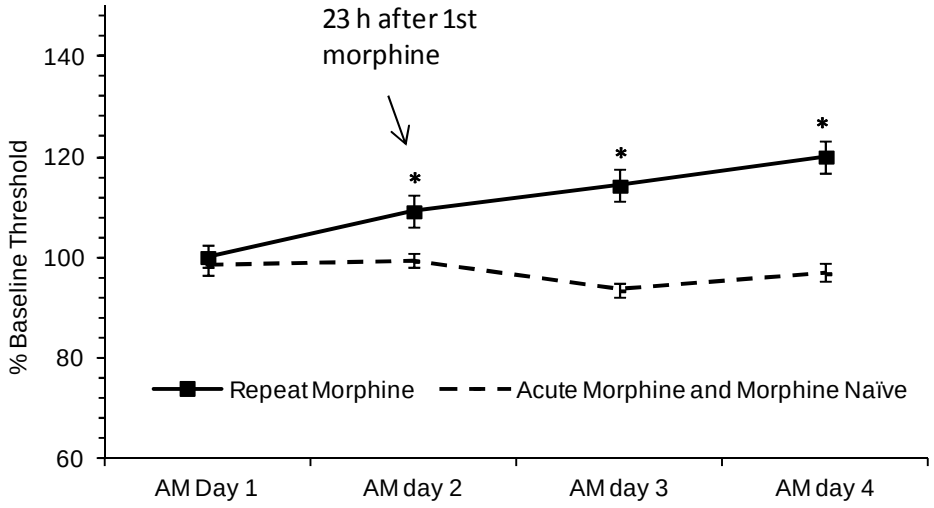


Figure 5. Effects of a morphine administration on thresholds measured 23 hrs after injection compared to rats not yet introduced to morphine. Repeat Morphine rats received the first SC injection after the AM day 1 run and after each AM run thereafter. Acute Morphine rats did not receive morphine until after the AM day 4 run. Data represents average threshold (±S.E.M.) (P < .001; significant main effect of morphine repeat vs. acute and vehicle in a between subjects analysis).

As seen in Figure 6, an acute dose of the highest tested naloxone concentration, 1.0 mg/kg elicited no significant change in Morphine-Naïve rats (Vehicle-Nal 1.0) as compared to the Vehicle-Vehicle control group [F(1,17)=1.61, P=.22], thus,

ruling out any effect of naloxone on reward thresholds in the absence of morphine pretreatment. Moreover, neither Acute or Repeat Morphine conditions produced any detectable change in reward thresholds when Vehicle instead of naloxone was administered 4 hr post-morphine (one-factor ANOVA comparing Vehicle-Vehicle, Acute Morphine-Vehicle, and Repeat-Morphine-Vehicle conditions, [$F(2,31)=1.50$, $P=.238$]).

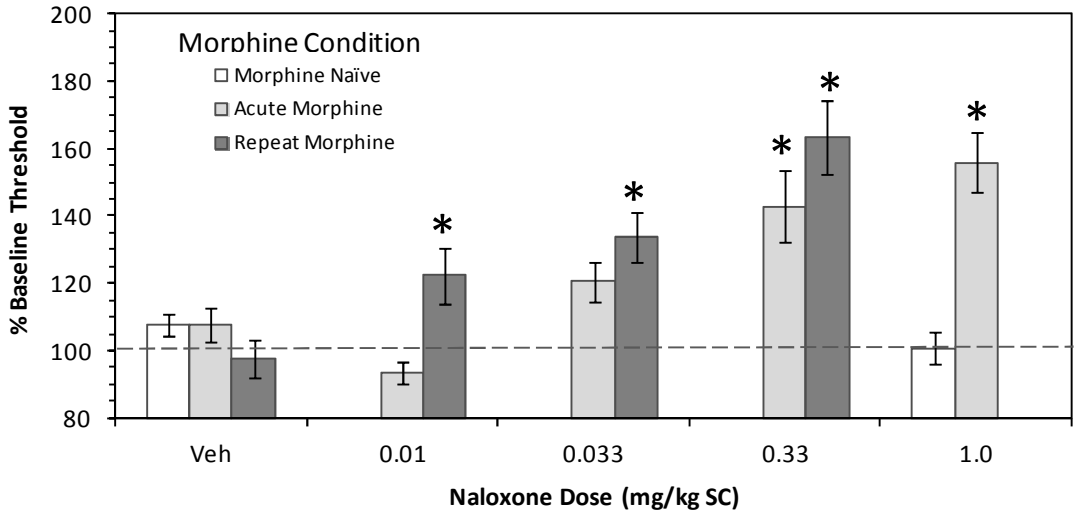


Figure 6. Effects of repeat and acute morphine exposure after naloxone precipitation on brain reward threshold. During testing days 1 to 3, rats received post-run morphine (Repeat) or saline (Acute). On day 4, rats received a SC injection of morphine post-run, which was followed 4 hours later by various doses of naloxone (0.01, 0.033, 0.33, 1.0 mg/kg) before the testing session. Control/vehicle rats were administered saline instead of morphine or naloxone. Data represents mean percent of baseline threshold (± S.E.M). *$P<.05$ significantly different from the controlled threshold measured under the same morphine condition.

Regardless of morphine condition, administering naloxone 4 hr following morphine significantly elevated brain reward thresholds in a dose-dependent fashion (Figure 6) (Main Effect of Naloxone Dose, Acute Morphine: [$F(1,58)=13.07$, $P=.0006$]; Main Effect of Naloxone Dose, Repeat Morphine:

[*F*(1,60)=8.13, *P*=.006]). Under the Acute Morphine condition, 0.33 mg/kg of naloxone was the lowest significant dose compared Acute Morphine-Vehicle condition, but greater effects were seen with higher doses (*P*< 0.05, Bonferroni-corrected) rats. Under Repeat Morphine Conditions, naloxone doses as low as 0.01 mg/kg significantly elevated reward thresholds, indicated a 30-fold shift in minimum effective dose of naloxone from Acute to Repeat Morphine conditions. When comparing Acute and Repeat Morphine conditions across the overlapping range of naloxone doses used in both groups (0.01, 0.033, 0.33 mg/kg), no significant interaction was observed between Morphine Condition and Naloxone Dose, suggesting a parallel shifts in the naloxone dose-effect function for precipitation of brain reward threshold elevations [*F*(2,60)=.43, *P*=.655] (Figure 6).

3.2 Experiment 2: CRF- R1 Antagonist Effects in CeA and NAC

In comparing AM and PM baselines across all treatment conditions (data not shown), a three-way mixed design ANOVA with treatment condition and brain region (CeA, NAC) as between-subjects factors and test session (AM vs. PM baseline threshold) as a repeated measure revealed no significant main effects of treatment or brain region or their interaction [*F*(4,97)=.21, *P* > .92]. As seen in Experiment 1, there was a modest but significant increase in threshold from AM to PM baseline [*F*(1,97)=55.07, *P* < .0001], but this did not differ among groups, as there was no significant treatment condition x test session interaction [*F*(1,97)=6.01, *P* > .015].

Before analyzing the results with naloxone-precipitated withdrawal and antalarmin reversal, a two-way ANOVA analysis with all control treatment conditions (Veh-Veh-Veh, Veh-Veh-Nal, Veh-Ant 3.3-Nal, and Mor-Veh-Veh) and brain region (CeA, NAC) as the factors was conducted. This analysis showed no overall significant main effect of treatment [$F(3,72) = 1.03$, $P > 0.38$], main effect of brain region [$F(1,72) = 1.63$, $P > 0.20$], or Brain Region × Treatment Condition interaction [$F(3,72)= 0.39$, $P > 0.75$] (Figure 7). Perhaps most importantly, these results indicate there was no non-specific effect of antalarmin infusion into either CeA or NAC in rats that were not pretreated with morphine (Veh-Ant 3.3-Nal vs. Veh-Veh-Veh or Veh-Veh-Nal).

As shown in Figure 7 A, in the absence of antalarmin infusion there was a highly significant increase in reward thresholds when naloxone (1.0 mg/kg) followed morphine (10 mg/kg), with a threshold increase to about 161% (Mor-Veh-Nal), as compared to an acute pretreatment of morphine with vehicle in place of naloxone (Mor-Veh-Veh). A one-way ANOVA with treatment condition (Mor-Veh-Veh, Mor-Veh-Nal, Mor-Ant 1.0-Nal, Mor-Ant 3.3-Nal) revealed a significant effect [$F(3,76)=11.14$, $P < .0001$]. Follow-up comparisons of individual groups revealed a significant increase in threshold when naloxone instead of vehicle was injected after morphine (Mor-Veh-Veh vs. Mor-Veh-Nal, $p < 0.05$ Bonferroni-corrected), and this threshold increase was significantly attenuated by 3.3 nmol antalarmin (Mor-Ant 3.3-Nal vs. Mor-Veh-Nal, $p < 0.05$; Mor-Ant 3.3-Nal vs. Mor-Veh-Veh, $p >0.38$). The diffusion control CeA group, with injector

termination 1 mm more shallow than the target site, showed no significant attenuation of naloxone-precipitated threshold increased when 3.3 nmol of antalarmin was infused (Diffusion Control vs. Mor-Veh-Nal, P > .85; Diffusion Control vs. Mor-Ant 3.3-Nal, P < 0.05 Bonferroni-corrected]. Therefore, diffusion of antalarmin up the injector tract did not appear to be a factor in the attenuation of brain reward deficits produced by antalarmin infused into the CeA.

As shown in Figure 7 B, in the absence of antalarmin infusion there was also a significant increase in reward thresholds when naloxone (1.0 mg/kg) followed morphine (10 mg/kg) (Mor-Veh-Nal), as compared to an acute pretreatment of morphine with vehicle in place of naloxone (Mor-Veh-Veh). A one-way ANOVA with treatment condition (Mor-Veh-Veh, Mor-Veh-Nal, Mor-Ant 1.0-Nal, Mor-Ant 3.3-Nal) revealed a significant effect [$F(3,38)=3.80$, $P > .01$]. Follow-up comparisons of individual groups revealed a significant increase in threshold when naloxone instead of vehicle was injected after morphine (Mor-Veh-Veh vs. Mor-Veh-Nal, $P < 0.05$ Bonferroni-corrected), and this threshold increase was significantly attenuated by 3.3 nmol antalarmin (Mor-Ant 3.3-Nal vs. Mor-Veh-Nal, P < 0.05; Mor-Ant 3.3-Nal vs. Mor-Veh-Veh, P >0.40). The diffusion control NAC group (NAC core), with injector termination 1 mm more shallow than the target site, showed no significant attenuation of naloxone-precipitated threshold increased when 3.3 nmol of antalarmin was infused (NAC core vs. Mor-Veh-Nal, P > .21), nor was there a significant difference from groups

with antalarmin directly infused into the NAC shell (NAC core vs. Mor-Ant 3.3-Nal [$F(1,37)=.99$, $P > 0.32$])

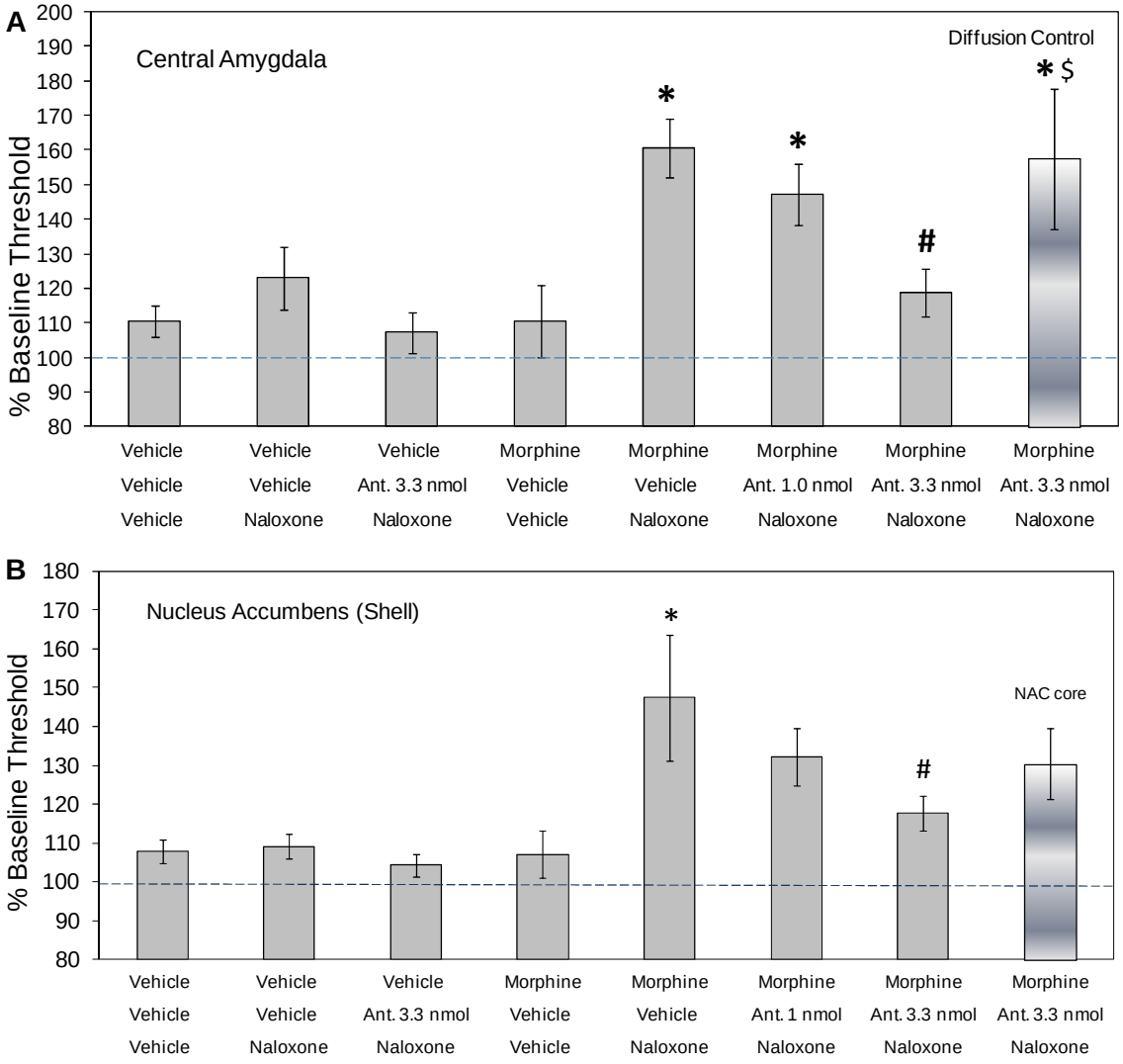


Figure 7. Effects of Antalarmin infusion in CeA and NAC prior to naloxone precipitation of acute premorphine administration on brain reward threshold. All rats received a SC injection of morphine (10 mg/kg). After 3 hr 45 minutes, rats received an IC administration of Antalarmin (1.0 or 3.3 nmol). After another 15 min, SC naloxone (1.0 mg/kg) was administered before testing the brain reward threshold. The last column represents antalarmin infusion into the diffusion control site. Data represents mean (± S.E.M) percent of baseline threshold for the CeA [A] and NAC shell [B]. * shows significant difference from Morphine-Vehicle-Vehicle groups. # significant difference from Morphine-Vehicle-Naloxone group. $ significant difference from Morphine-Antalarmin-Naloxone group that directly targeted antalarmin in the region of interest. Significant where $P<0.05$.

4. Discussion

4.1 Experiment 1: Naloxone Dose Response Determination

As supported by previous studies (Liu and Schulteis, 2004), results of Experiment 1 confirmed that in rats pretreated with Acute or Repeat morphine (10 mg/kg), naloxone dose-dependent increased brain reward thresholds as measured by ICSS. Naloxone, *per se* (Vehicle-Naloxone), did not produce significant changes in threshold if not paired with morphine exposure, which is consistent with a study be Perry et al. (1981) that failed to show an effect on threshold in an ICSS session as a result of even very high doses of naloxone (16 mg/kg). After morphine 10 mg/kg pretreatment(s), sensitivity to naloxone was detectable at doses as low as 0.01 mg/kg after repeated morphine exposure, and as low as 0.33 mg/kg of naloxone in Acute Morphine rats. Acute opioid dependence, as first observed through the work of Wikler and Carter (1953), has been replicated in this study to show a brain reward deficit from naloxone-precipitation of withdrawal from an acute dose of morphine. Repeat Morphine administration potentiated the reward deficits produced by precipitated withdrawal, producing a parallel shift in naloxone potency to the left of at least 30-fold relative to its potency in the Acute Morphine group.

Of particular interest from this study is the large average threshold increase (56%) from baseline values after naloxone 1.0 mg/kg precipitation from an acute exposure to 10 mg/kg of morphine. This can be compared to previous

dose-response studies using *5.6 mg/kg* of morphine in which animals showed only a 35% maximal increase in baseline thresholds following Acute Morphine (Liu and Schulteis, 2004). The stronger contrast from baseline values using 10 mg/kg of morphine will facilitate studies such as those conducted in Experiment 2, where one seeks to reverse the naloxone-precipitated withdrawal effect by treatments aimed to elucidate the neural mechanisms mediating acute opioid dependence and withdrawal.

The modest, but significant threshold increase observed 23 h after the first morphine treatment (see AM threshold increases on Days, 2, 3 and 4 in Repeat Morphine group, Figure 4), but not 4 h after Acute or Repeat Morphine treatment (Morphine-Veh-Veh in Figures 6-7), may be attributed to a measurable spontaneous withdrawal state that emerges between 4 and 23 hr post-morphine, and replicates a similar observation by Liu and Schulteis (2004).

4.2 Experiment 2: CRF- R1 Antagonist Effects in CeA and NA

Results from this study showed a site-specific interaction between CRF-R1 antagonism in regions of the extended amygdala and precipitated opioid withdrawal. The CRF-R1 antagonist, antalarmin, applied to the CeA and NAC just prior to naloxone-precipitation successfully attenuated elevations in brain reward thresholds linked to withdrawal. The finding expands on previous findings that CRF antagonists suppress some of the negative states of opioid withdrawal (Heinrichs et al., 1995a; Stinus et al., 2005), at doses of antagonist that have no

effect on brain reward when the subjects is not “stressed” by withdrawal. Targeting CRF-R1 receptors in the extended amygdala as the neural circuit associated with drug addiction may provide an important link to developing a drug that has the potential to block the dysphoric states associated with opioid withdrawal and prevent the addictive properties of opioids and possibly other drugs of abuse. The null results from antalarmin infusion into a diffusion control site (1 mm dorsal to the CeA and NAC shell) proved the site specific activity of the injected CRF-R1 antagonist.

The central amygdala mediates both fear-like and avoidance behaviors, and also contains CRF receptors that play a role in the expression of stress-induced behaviors (Swiergiel et al., 1993). Previous studies show that CRF antagonists infused into the CeA effectively block the robust motivational effects of precipitated opioid withdrawal associated with place aversion (Heinrichs et al., 1995b; Schulteis et al., 1994) and reversed anxiogenic effects of ethanol withdrawal (Rassnick et al., 1993). The results from this study show a significant attenuation of reward threshold upon antalarmin administration in rats with reward thresholds dropping from 161% to 119% of baseline.

Criner et al. (2007) had previously established that an infusion of the opioid antagonist methylnaloxonium, into the general NAC region (at the border of the core and shell) suppressed operant responses to food reward after an acute 5.6 mg/kg morphine injection, indicating a role for opioid receptors in the NAC in mediating this aversive precipitated withdrawal response from acute

opioid dependence. In this study, a distinction was established between the core and shell of the NAC for the effects of antalarmin on brain reward thresholds during withdrawal. The NAC shell was the primary target of the study because the shell more than the core shares similarities with other components of the extended amygdala (Zahm, 2006). The NAC shell also appears to express a much higher dopamine increase upon opioid administration compared to the core region, which is thought to be important in reward and addiction behaviors (Pontieri et al., 1995). Due to the location of the NAC shell and surgery target, the diffusion control target for NAC shell happened to fall in the NAC core, so the NAC core controlled for diffusion effects as well as a test site for its specific antalarmin sensitivity. While infusion of CRF-R1 antagonist into the main target, NAC shell, produced a significant decrease in brain reward thresholds from opioid precipitation, CRF-R1 antagonism in the NAC core caused minimal reversal effects. There was some non-significant attenuation of withdrawal-induced threshold elevations with antalarmin infusion into the core. The effects were neither significantly different from complete withdrawal (Mor-Veh-Nal group, Figure 6) nor from antarlarmin antagonism within the shell (Mor-Ant 3.3-Nal group), which may be attributed to some role for blockade of CRF receptors in the core region; alternately the modest non-significant effect observed in the core could be the result of diffusion ventrally to the shell region. Overall, the current data support the functional compartmentation of the NAC subregions and the significance of CRF receptors in the NAC shell during opioid withdrawal.

Individually, the CeA and the NAC shell are involved in the circuitry that inhibits some of the distressing qualities associated with acute drug withdrawal. One possible mechanism of a more complete suppression of opioid withdrawal effects may be the simultaneous antagonism of CRF-R1 at both sites. While many of the projections in these regions overlap, the different outputs may combine for a synergistic effect or enable a more complete reversal of brain reward deficits during drug withdrawal.

Another region in the extended amygdala important to drug response is the BNST, which was not examined in the current study. For example, studies report activation of the BNST in response to stress (Bonaz and Taché, 1994) and an increase in CRF levels upon drug withdrawal in this region (Olive et al., 2002). CRF-R1 antagonist administration in the BNST also has the potential to attenuate reward thresholds during opioid withdrawal. Future studies can also test whether a state of repeated morphine use activates recruitment of additional regions sensitive to CRF. In addition, better understanding of the downstream circuitry from the extended amygdala will help to elucidate the complete pathway involved in activating the drug sensitization response.

In this study, antalarmin intracerebrally infused into the brain exclusively targeted receptors at the location of interest. Selective CRF-R1 antagonists can effectively block anxiety-like and depression-like symptoms (Bale and Vale, 2004), including naloxone-precipitated morphine withdrawal, when administered systemically (Stinus et al., 2005) and anxiety-related behavior when centrally

infused (Henry et al., 2006). For example, in rats: subcutaneous (Shaham et al., 1998), intravenous (Gutman et al., 2003), and intraperitoneal (Schulz et al., 1996) routes reduced levels of stress-induced self administration of abused drugs, reduced central ACTH levels and blocked potentiation of acoustic startle, respectively. In primates, intravenous (Herod et al., 2011) and oral (Habib et al., 2000) routes successfully reduced stress-induced reproductive dysfunction and inhibited behaviors associated with anxiety during a social stressor. Drug companies are actively developing CRF-R1 specific antagonists that are small, lipophilic, and nonpeptidic, which allow them to cross the blood brain barrier to bind at all receptor locations, giving these drugs the potential to be centrally active after peripheral administration. Early human trials on CRF-R1 inactivation in clinically depressed patients observed successful reductions in depression and anxiety symptoms (Zobel et al., 2000). With better understanding of the stress circuitry, the antagonizism of CRF-R1 has significant novel potential in targeting the pharmacotherapeutic treatment of stress-related disorders, including addiction.

In summary, suppression of CRF-R1 binding in the extended amygdala decreases the brain reward deficits associated with acute opioid withdrawal. Whether a single region of the extended amygdala is responsible for the dysphoria-like effects accompanying opioid withdrawal, or whether a synergistic effect among multiple regions occurs, these results reinforce the significance of the extended amygdala in activating the stress system involved with opioid

withdrawal. Opioid effects may be applied to many other drugs of abuse that share a common neural circuitry for reward. These results suggest that CRF decreases brain reward during times of drug abstinence, and may have a general motivating effect of increasing drug intake in order to overcome the negative emotional consequences associated with the activation of the CRF stress system. The data indicate that recruitment of CRF systems is a rapid neuroadaptive stress-like response seen during withdrawal from the very first dose of opioid. Therefore targeting and understanding the negative motivational aspects of addiction may provide a key link to understanding an individual's transition from casual to compulsive drug use.

Works Cited

Alheid, G. F., de Olmos, J. S., and Beltramino, C. A. (1995). The rat nervous system, 2nd edn (San Diego: Academic Press).

Alheid, G. F., and Heimer, L. (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: The striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience *27*, 1-39.

Azar, M., Jones, B., and Schulteis, G. (2003). Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. Psychopharmacology *170*, 42-50.

Azorlosa, J., Stitzer, M., and Greenwald, M. (1994). Opioid physical dependence development: effects of single versus repeated morphine pretreatments and of subjects' opioid exposure history. Psychopharmacology *114*, 71-80.

Bale, T. L., and Vale, W. W. (2004). CRF AND CRF RECEPTORS: Role in Stress Responsivity and Other Behaviors. Annual Review of Pharmacology and Toxicology *44*, 525-557.

Bonaz, B., and Taché, Y. (1994). Induction of Fos immunoreactivity in the rat brain after cold-restraint induced gastric lesions and fecal excretion. Brain Research *652*, 56-64.

Bruijnzeel, A. W., Prado, M., and Isaac, S. (2009). Corticotropin-releasing factor-1 receptor activation mediates nicotine withdrawal-induced deficit in brain reward function and stress-induced relapse. Biol Psychiatry *66*, 110-117.

Bruijnzeel, A. W., Small, E., Pasek, T. M., and Yamada, H. (2010). Corticotropin-releasing factor mediates the dysphoria-like state associated with alcohol withdrawal in rats. Behav Brain Res *210*, 288-291.

Chalmers, D., Lovenberg, T., and De Souza, E. (1995). Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. The Journal of Neuroscience *15*, 6340-6350.

Criner, S. H., Liu, J., and Schulteis, G. (2007). Rapid neuroadaptation in the nucleus accumbens and bed nucleus of the stria terminalis mediates suppression of operant responding during withdrawal from acute opioid dependence. Neuroscience *144*, 1436-1446.

Dunn, A. J., and Berridge, C. W. (1990). Physiological and behavioral responses

to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res Brain Res Rev *15*, 71-100.

Easterling, K. W., Plovnick, R. M., and Holtzman, S. G. (2000). Acute opioid but not benzodiazepine dependence in rats responding for intracranial self-stimulation. Psychopharmacology *148*, 263-271.

Funk, C. K., O'Dell, L. E., Crawford, E. F., and Koob, G. F. (2006). Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. J Neurosci *26*, 11324-11332.

Gutman, D. A., Owens, M. J., Skelton, K. H., Thrivikraman, K. V., and Nemeroff, C. B. (2003). The corticotropin-releasing factor1 receptor antagonist R121919 attenuates the behavioral and endocrine responses to stress. J Pharmacol Exp Ther *304*, 874-880.

Heimer, L., and Alheid, G. F. (1991). Piecing together the puzzle of basal forebrain anatomy. Adv Exp Med Biol *295*, 1-42.

Heinrichs, S. C., Menzaghi, F., Merlo Pich, E., Britton, K. T., and Koob, G. F. (1995a). The role of CRF in behavioral aspects of stress. Ann N Y Acad Sci *771*, 92-104.

Heinrichs, S. C., Menzaghi, F., Schulteis, G., Koob, G. F., and Stinus, L. (1995b). Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. Behav Pharmacol *6*, 74-80.

Henry, B., Vale, W., and Markou, A. (2006). The effect of lateral septum corticotropin-releasing factor receptor 2 activation on anxiety is modulated by stress. Journal of Neuroscience *26*, 9142-9152.

Herod, S. M., Pohl, C. R., and Cameron, J. L. (2011). Treatment with a CRH-R1 antagonist prevents stress-induced suppression of the central neural drive to the reproductive axis in female macaques. Am J Physiol Endocrinol Metab *300*, E19-27.

Johnson, P. M., Hollander, J. A., and Kenny, P. J. (2008). Decreased brain reward function during nicotine withdrawal in C57BL6 mice: evidence from intracranial self-stimulation (ICSS) studies. Pharmacol Biochem Behav *90*, 409-415.

Koob, G. F. (2006). The neurobiology of addiction: a neuroadaptational view relevant for diagnosis. Addiction *101 Suppl 1*, 23-30.

Koob, G. F. (2008). A role for brain stress systems in addiction. Neuron *59*, 11-34.

Koob, G. F., and Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. Science *278*, 52-58.

Koob, G. F., and Le Moal, M. (2006). Neurobiology of addiction, (Amsterdam ; Boston: Elsevier/Academic Press).

Kornetsky, C., and Bain, B., eds. (1990). A model for drug-induced euphoria, (Oxford, England: Wiley, John & Sons, Inc.).

Kornetsky, C., and Esposito, R. U. (1979). Euphorigenic drugs: effects on the reward pathways of the brain. Fed Proc *38*, 2473-2476.

Lin, D., Koob, G. F., and Markou, A. (1999). Differential effects of withdrawal from chronic amphetamine or fluoxetine administration on brain stimulation reward in the rat--interactions between the two drugs. Psychopharmacology (Berl) *145*, 283-294.

Liu, J., and Schulteis, G. (2004). Brain reward deficits accompany naloxone-precipitated withdrawal from acute opioid dependence. Pharmacol Biochem Behav *79*, 101-108.

Lonergan, C., Gutierrez-Reed, N., Archer, C., Koob, G. F., Zorrilla, E. P., Powell, S. B., and Schulteis, G. (submitted). Brain reward deficits resulting from naloxone-precipitated opioid withdrawal are reversed by selective antagonism of CRF type-1 receptors.

Markou, A., and Koob, G. F. (1991). Postcocaine anhedonia. An animal model of cocaine withdrawal. Neuropsychopharmacology *4*, 17-26.

Markou, A., and Koob, G. F. (1992). Construct validity of a self-stimulation threshold paradigm: effects of reward and performance manipulations. Physiol Behav *51*, 111-119.

Martin, W. R., and Eades, C. G. (1964). A COMPARISON BETWEEN ACUTE AND CHRONIC PHYSICAL DEPENDENCE IN THE CHRONIC SPINAL DOG. Journal of Pharmacology and Experimental Therapeutics *146*, 385-394.

Mills, K. L., Teesson, M., Ross, J., and Peters, L. (2006). Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. Am J Psychiatry *163*, 652-658.

Newport, D. J., and Nemeroff, C. B. (2000). Neurobiology of posttraumatic stress disorder. Curr Opin Neurobiol *10*, 211-218.

Olive, M. F., Koenig, H. N., Nannini, M. A., and Hodge, C. W. (2002). Elevated extracellular CRF levels in the bed nucleus of the stria terminalis during ethanol

withdrawal and reduction by subsequent ethanol intake. Pharmacol Biochem Behav *72*, 213-220.

Parker, L. A., and Joshi, A. (1998). Naloxone-precipitated morphine withdrawal induced place aversions: effect of naloxone at 24 hours postmorphine. Pharmacol Biochem Behav *61*, 331-333.

Paulozzi, L. J., Jones, C. M., Mack, K. A., and Rudd, R. A. (2011). Vital signs: overdoses of prescription opioid pain relievers --- United States, 1999--2008. MMWR Morb Mortal Wkly Rep *60*, 1487-1492.

Paxinos, G., and Watson, C. (2007). The Rat Brain in Stereotaxic Coordinates, 6 edn (San Diego: Academin Press).

Perry, W., Esposito, R. U., and Kornetsky, C. (1981). Effects of chronic naloxone treatment on brain-stimulation reward. Pharmacol Biochem Behav *14*, 247-249.

Pontieri, F. E., Tanda, G., and Di Chiara, G. (1995). Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. Proc Natl Acad Sci U S A *92*, 12304-12308.

Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P. E., and Vale, W. (1994). Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc Natl Acad Sci U S A *91*, 8777-8781.

Rassnick, S., Heinrichs, S. C., Britton, K. T., and Koob, G. F. (1993). Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Research *605*, 25-32.

Richter, R. M., and Weiss, F. (1999). In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. Synapse *32*, 254-261.

Sarnyai, Z., Shaham, Y., and Heinrichs, S. C. (2001). The role of corticotropin-releasing factor in drug addiction. Pharmacol Rev *53*, 209-243.

Schulteis, G. (2010a). Acute Dependence. In Encyclopedia of Behavioral Neuroscience, F.K. Editors-in-Chief: George, M. Michel Le, M.L.M. Richard F. ThompsonA2 - Editors-in-Chief: George F. Koob, and F.T. Richard, eds. (Oxford: Academic Press), pp. 10-19.

Schulteis, G. (2010b). Brain Stimulation and Addiction. In Encyclopedia of Behavioral Neuroscience, F.K. Editors-in-Chief: George, M. Michel Le, M.L.M.

Richard F. ThompsonA2 - Editors-in-Chief: George F. Koob, and F.T. Richard, eds. (Oxford: Academic Press), pp. 209-220.

Schulteis, G., Markou, A., Cole, M., and Koob, G. F. (1995). Decreased Brain Reward Produced by Ethanol Withdrawal. P Natl Acad Sci USA *92*, 5880-5884.

Schulteis, G., Markou, A., Gold, L. H., Stinus, L., and Koob, G. F. (1994). Relative Sensitivity to Naloxone of Multiple Indexes of Opiate Withdrawal - a Quantitative Dose-Response Analysis. Journal of Pharmacology and Experimental Therapeutics *271*, 1391-1398.

Schulz, D. W., Mansbach, R. S., Sprouse, J., Braselton, J. P., Collins, J., Corman, M., Dunaiskis, A., Faraci, S., Schmidt, A. W., Seeger, T.*, et al.* (1996). CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. Proc Natl Acad Sci U S A *93*, 10477-10482.

Shaham, Y., Erb, S., Leung, S., Buczek, Y., and Stewart, J. (1998). CP-154,526, a selective, non-peptide antagonist of the cortisotropin-releasing factor(1) receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. Psychopharmacology *137*, 184-190.

Stinus, L., Cador, M., Zorrilla, E. P., and Koob, G. F. (2005). Buprenorphine and a CRF1 antagonist block the acquisition of opiate withdrawal-induced conditioned place aversion in rats. Neuropsychopharmacology *30*, 90-98.

Sullivan, M. D., Edlund, M. J., Steffick, D., and Unützer, J. (2005). Regular use of prescribed opioids: Association with common psychiatric disorders. Pain *119*, 95-103.

Swiergiel, A. H., Takahashi, L. K., and Kalin, N. H. (1993). Attenuation of Stress-Induced Behavior by Antagonism of Corticotropin-Releasing Factor Receptors in the Central Amygdala in the Rat. Brain Research *623*, 229-234.

Van Pett, K., Viau, V., Bittencourt, J. C., Chan, R. K., Li, H. Y., Arias, C., Prins, G. S., Perrin, M., Vale, W., and Sawchenko, P. E. (2000). Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol *428*, 191-212.

Veinante, P., Stoeckel, M. E., Lasbennes, F., and Freund-Mercier, M. J. (2003). c-Fos and peptide immunoreactivities in the central extended amygdala of morphine-dependent rats after naloxone-precipitated withdrawal. Eur J Neurosci *18*, 1295-1305.

Wikler, A., and Carter, R. L. (1953). Effects of single doses of N-Allylnormorphine on hindlimb reflexes of chronic spinal dogs during cycles of morphine addiction. J Pharmacol Exp Ther *109*, 92-101.

Zahm, D. S. (2006). The evolving theory of basal forebrain functional-anatomical 'macrosystems'. Neurosci Biobehav Rev *30*, 148-172.

Zobel, A. W., Nickel, T., Kunzel, H. E., Ackl, N., Sonntag, A., Ising, M., and Holsboer, F. (2000). Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J Psychiat Res *34*, 171-181.

Zorrilla, E. P., and Koob, G. F. (2004). The therapeutic potential of CRF1 antagonists for anxiety. Expert Opin Inv Drug *13*, 799-828.