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Central nervous system corticotropin releasing factor (CRF) systems contribute to increased anxiety-like behavior during opioid withdrawal : an analysis of neuroanatomical substrates

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Central Nervous System Corticotropin Releasing Factor (CRF) Systems Contribute to Increased Anxiety-Like Behavior During Opioid Withdrawal: An Analysis of Neuroanatomical Substrates

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

in

Biology

by

Alicia Trigeiro

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Professor Gerhard Schulteis, Chair
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Professor Shelley Halpain

2011

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

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
Ant	Antalarmin
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
CeA	Central Amygdala
CRF	Corticotropin Releasing Factor
Diff. Cont.	Diffusion Controls
HPA	Hypothalamic-pituitary-adrenal
Mor	Morphine
NAC	Nucleus Accumbens
SC	Subcutaneous
Veh	Vehicle

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ABSTRACT OF THE THESIS

Central Nervous System Corticotropin Releasing Factor (CRF) Systems Contribute to Increased Anxiety-Like Behavior During Opioid Withdrawal: An Analysis of Neuroanatomical Substrates

by

Alicia Trigeiro

Master of Science in Biology

University of California, San Diego, 2011

Professor Gerhard Schulteis, Chair
Professor Massimo Scanziani, Co-Chair

Increased release of the stress neurotransmitter corticotropin-releasing factor (CRF) in the extended amygdala mediates ethanol withdrawal signs such as anxiety-like behaviors. The present study determined whether anxiety-like consequences of naloxone-precipitated withdrawal from morphine dependence also involves CRF hyperactivity in the extended amygdala, by selectively infusing antalarmin, an antagonist that selectively blocks CRF 1 receptors, into the nucleus accumbens shell (NAC) or the central amygdala (CeA). Anxiety-like behavior was assessed as a reduction in willingness to explore the open arms of an elevated plus maze. Male Wistar rats were implanted with bilateral cannulae in the NAC shell or the CeA. Dependence was induced by daily injections of 10

mg/kg of morphine over a period of 4 days (non-dependent controls received vehicle in place of morphine). Eight hours after morphine injection on the fourth day, withdrawal was precipitated by naloxone (0.33 mg/kg; controls received vehicle in place of naloxone. Bilateral infusion of the CRF-1 receptor antagonist antalarmin (1.0, 3.3, or 10.0nmol) or vehicle preceded naloxone by 10 min. Naloxone administered after morphine but not vehicle reduced the relative time spent in, and number of entries into, the open vs. closed arms of the maze. Antalarmin dose-dependently attenuated these morphine withdrawal anxiety-like signs when infused in the NAC shell but not CeA. The same dose of intra-NAC antalarmin that attenuated anxiety-like signs antalarmin reduced activity (number of closed arm entries), suggesting that blunting CRF's arousing effects in the NAC may result in a reduction of morphine withdrawal anxiety.

1. Introduction

Drug addiction is a growing and serious problem in the United States. Healthcare costs associated with drug addiction continue to soar and cost the United States billions of dollars a year in hospital stays, treatment, and addiction management (Office of National Drug Control Policy, 2004). As yet, there have been few successful treatments developed to help prevent relapses with drug addiction. Addiction can be defined as "compulsive drug use despite negative consequences" including failure in life roles, medical problems, risk of injury and trouble with the law (Hyman 2005). Although some people are able to stop the compulsive use of drugs, many individuals relapse and continue a pattern of chronic addiction.

Many individuals are not able to maintain abstinence in part because the symptoms associated with withdrawal are extremely unpleasant and difficult to overcome. Withdrawal symptoms from opioid dependence can include a variety of autonomic, somatic, and emotional symptoms such as agitation, anxiety, nausea, diarrhea, dysphoria, sweating, and irritability (Haertzen and Hooks, 1969; O'Brien et al. 1976). The negative emotional withdrawal symptoms such as anxiety and dysphoria/depression are often the causes of relapse after periods of abstinence, maintenance, and escalation to compulsive drug use (Koob, 2008). Thus a better understanding of the effects of withdrawal on measures of anxiety and mood both clinically and in animal models may aid in our understanding of the progression from use to abuse and help identify mechanisms associated with drug abuse and relapse.

Investigators have studied various proposed physiological mechanisms of drug addiction and it is now believed that drug addiction takes over the reward pathway, as well as the pathways involved in learning and memory (Hyman, 2005). In normal individuals, the reward pathway is responsible for the maintenance of certain motivational states, such as eating or drinking. If a drug hijacks this system, the methods used by the body to survive and maintain homeostasis, such as eating, drinking, etc., are pushed aside for the chance to activate the reward pathway when using that particular drug. One of the reasons that withdrawal is so hard to overcome is because the drug causes a disruption within the reward pathway and becomes the source of motivation for the individual to keep satisfying the urge to abuse the drug.

Corticotropin-releasing factor (CRF) is an important regulator in the endocrine stress response. CRF belongs to a family of neuropeptides (e.g. corticotrophin-releasing hormone, urocortin) and its receptors have endogenous functions, including regulation of food intake, vasoregulation, thermoregulation, growth and metabolism, reproduction, and physiological effects on stress and anxiety (Lovejoy and Balment, 1999). CRF receptors are G protein-coupled receptors and come in two types, CRF-1 receptors and CRF-2 receptors (Vale and Bale, 2004). CRF-1 receptors are distributed throughout the cerebral cortex, cerebellum, olfactory bulb, medial septum, hippocampus, amygdala, basal forebrain, and pituitary (Bale and Vale, 2004). CRF ligands have been implicated in the stress response and its associated behaviors (Lovejoy and Balment, 1999). During a stressful event, CRF activates the hypothalamic-pituitary-adrenal (HPA) axis, acting at CRF-1 receptors on the anterior pituitary corticotropes to stimulate the release of adrenocorticotropic hormone (ACTH). The receptors in the adrenal gland are then

stimulated by ACTH, which causes the release of glucorticoids (e.g. corticosterone). In addition to its role in stimulating the HPA axis, CRF also acts at extrahypothalamic sites within the brain to regulate anxiety-like behavior (Risbrough and Stein, 2006). Depression has been linked to the neurocircuitry involving dysfunctional CRF actions and regulators (Nemeroff, 1988). CRF-1 receptor activation is a key component in the development of anxiety (Kehne, 2007; Koob and Heinrichs, 1999) and recent studies have shown that CRF-1 receptor antagonists may potentially be used in the future as a treatment for anxiety disorders (Vale and Bale, 2004). CRF antagonists have been shown to decrease the stress response in rodents, and are also implicated in addiction, where CRF antagonists can block stress-induced increases in self-administration of drugs (Koob and Zorrilla, 2010).

The extended amygdala is a critical component of brain reward circuitry, and adaptive responses to chronic drug intake in the extended amygdala is thought to be one of the primary substrates for emotional signs of drug (Koob and Le Moal, 2008). The extended amygdala has been shown to be a common anatomical substrate that is active during acute drug administration and helps drive the negative effects of drug addition that occur as well, such as dysphoria and anxiety (Koob and Zorrilla, 2010). The extended amygdala includes the central nucleus of the amygdala (CEA), the bed nucleus of the stria terminalis (BNST), and the shell of the nucleus accumbens (NAC). All of these structures of the extended amygdala share similar neurocircuitry and have a high density of cell bodies and terminals for CRF (Koob and Zorrilla, 2010). Two areas within the extended amygdala, the CeA and the shell of the NAC, are central to the negative reinforcement that is associated with morphine dependence and withdrawal (Criner and

Schluteis, 2007). Emotional withdrawal symptoms, such as dysphoria and increased anxiety, involve CRF and norepinephrine in the extended amygdala. For example, ethanol withdrawal produces anxiety-like behavior and increased CRF release in the CeA (Merlo Pich et al., 1995).

One of the most common responses to acute and chronic withdrawal is the expression of anxiety-like behavior, which can be operationalized in rodents using tests such as the open field, elevated plus maze, defensive withdrawal, and social interaction test (Covington and Miczek, 2003; Fendt and Mucha, 2001; Kantak and Miczek, 1986; Schulteis et al., 1994;). Anxiety-like behavior in most of these paradigms can be reversed by the administration of a CRF antagonist (Koob and Zorrilla, 2010). Antalarmin is a non-peptide drug that blocks CRF-1 receptors at the level of the hypothalamus and reduces the levels of ACTH that are released in response to stress. It also acts at extrahypothalamic sites to affect anxiety-like behavior (Marinelli et al., 2007). For example, the CRF-1 receptor antagonist, antalarmin, decreased conditioned fear responses and increased open-arm time in an elevated plus maze (Bale and Vale, 2004; Lundkvist et al., 1996). Antalarmin has also been shown to reduce withdrawal symptoms from chronic opioid use, specifically blocking place aversion, which was produced by precipitated morphine withdrawal (Stinus et al., 2005).

In rodents either spontaneous withdrawal or precipitated withdrawal from opioids can be measured in the laboratory. Spontaneous withdrawal refers to withdrawal symptoms appearing at certain time points after the last drug administration and may be unpredictably observed following cessation of opioid treatment (Schulteis, 2010). Precipitated withdrawal involves administering a competitive antagonist that competes

with the drug of abuse for access to receptors and hence acutely blocks the effects of the drug. For example, competitive antagonists, such as naloxone, can rapidly elicit withdrawal symptoms from opioid agonists (Schulteis et al., 2009). The precipitated withdrawal provides a time window where withdrawal symptoms can be readily observed. In animal studies, intervals of 2-12 hours between morphine and naloxone administration typically produces the most intense withdrawal signs (Schulteis, 2010). Spontaneous withdrawal symptoms, on the other hand, may be observed unpredictably and irregularly following the last opioid treatment. The symptoms observed during spontaneous withdrawal versus precipitated withdrawal are very similar, making precipitated withdrawal the preferred method to study opioid dependence. Many studies involving spontaneous and precipitated withdrawal have been used to study anxiety-like behavior, many using the elevated plus maze (e.g. Schulteis et al, 1998).

In this thesis, the link between the CRF system within the extended amygdala and the anxiety-like symptoms produced by opioid withdrawal were examined. The CeA and NAC regions of the extended amygdala have large numbers of CRF-1 receptors and by using a CRF antagonist, antalarmin, we tested whether these regions are responsible for the withdrawal symptoms that occur during opioid dependence. We measured anxiety-like behavior during withdrawal in the elevated plus maze, a common test of anxiety-like behavior in rodents.

The elevated plus maze has been used to evaluate different types of anxiety-like behavior in animals. It is an excellent tool to study anxiety-like behaviors because it does not require extensive conditioning, training, or aversive stimulation. The elevated plus maze is a plus-shaped apparatus with two open and two closed arms, each with an open

roof, that is elevated two to three feet off the floor (see Figure 5). The test involves one of the animal's innate behaviors, which is wanting to explore all parts of a novel environment, in conflict with a fear of open spaces (i.e. the open arms). The elevated plus maze produces an approach-avoidance conflict, which produces anxiogenic effects in the animal. The behavior of the rats can then be measured in terms of how willing they are to explore the open arms of the plus maze and how much time they spend in the closed or open arms (Lapiz-Bluhm et al., 2008).

In this study, the drug naloxone has been used to precipitate morphine-induced withdrawal symptoms. Naloxone acts as a competitive antagonist predominantly on μ opioid receptors, reversibly binding to prevent drugs such as morphine, a μ opioid agonist, from activating the receptor (Brunton, 2010). By itself naloxone exhibits little pharmacological activity in the absence of opioids (Chamberlain, 1994). The interaction between naloxone and morphine provided a mechanism for us to evaluate the anxiety-like behavior produced by withdrawal symptoms in a controlled manner.

The elevated plus maze is used to measure anxiety-like behavior produced by both the spontaneous and naloxone-precipitated withdrawal models (Schulteis et al., 1998). Naloxone is considered to be a reliable method for producing precipitated morphine withdrawal symptoms within a set time course and causes a decrease in the amount of time spent in the open arms and the percentage of open arm entries. It results in a significant dose-dependent expression of anxiety-like behavior and does not produce any changes to the general activity of the animal after morphine treatment (Zhang and Schulteis, 2008). The negative emotional signs associated with withdrawal are products

of the naloxone-precipitated withdrawal from opioid dependence and this model can be used to test for anxiety on a plus maze (Zhang and Schulteis, 2008).

2. Materials and Methods

2.1 Animals

Male Wistar rats (n=234, Harlan Labs, Indianapolis, IN) purchased at a weight of 220-225g were housed (2-3/cage) in a temperature and humidity-controlled room with a 12 hour light/dark cycle (lights on at 6am). Rats had *ad libitum* access to food and water at all times. At the time of surgery, the rats weighed between 280-330g and had been acclimated to the colony for at least one week. All experimental procedures were approved by the Subcommittee on Animal Studies of the VA San Diego Healthcare System, an AAALAC-accredited facility, and are in strict accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals" (Revised 1996). All efforts were made to minimize animal suffering and to reduce the number of animals required.

2.2 Drugs

Morphine sulfate (Research Resources Drug Supply System of the National Institute on Drug Abuse, Bethesda, MD, USA) was administered subcutaneously (SC) at a dose of 10mg/kg. Naloxone HCL (Sigma, St. Louis, MO, USA) was administered at a dose of 0.33 mg/kg. Both drugs were prepared using physiological saline (0.9%) and all injections were made subcutaneously in a volume of 0.1ml/100g body weight. Antalarmin hydrochloride (Sigma, St. Louis, MO, USA) 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 the dose infused into each hemisphere in a volume of 0.5μL per side. Antalarmin was infused into either the CeA or NAC at concentrations of 1.0nmol, 3.3nmol, or 10.0nmol of antalarmin.

2.2.1 Use of Antalarmin

Antalarmin is a non-peptide CRF-1 receptor antagonist and has been used to reduce the systemic release of ACTH in response to stress. Antalarmin acts on the HPA axis and the extrahypothalamic sites, such as the extended amygdala (Funk et al., 2006), by reversing CRF-mediated stress responses without causing other major side effects (Zoumakis et al., 2006).

2.3 Extended amygdala Bilateral Cannulation Surgeries

Rats were put under anesthesia by inhalation of 2-4% isoflurane and the surgical site on the head was shaved and cleaned with a combination of alcohol and betadine. The rats were then placed in a stereotaxic apparatus in order to implant the cannulae. The incisor bar was set to “flat skull” at a level of -3.3 mm. The cannulae (26 gauge, Plastics One, VA) used were 12.5 mm long, stainless steel, and were sterilized in betadine and 70% ethanol. Dental cement and stainless steel screws (Plastics One, VA) were used to

fix the cannulae to the skull. In order to avoid the obstruction of any part of the cannulae, a 12.5 mm stylet occluder was inserted into the cannulae following surgery and was cemented in place. It was kept there during recovery and removed right before infusions. Local analgesic and antibiotic ointment were dispensed to the site of the incision at the end of surgery and rats were allowed to recover for at least 5 days before being tested on the plus maze. Guide cannulae were inserted into the following regions of the extended amygdala: CeA and NAC.

2.3.1 Central Amygdala

Guide cannulae were aimed at the following coordinates of the Paxinos and Watson Rat Brain Atlas (6^{th} edition, 2007); relative to bregma -2.2mm anteroposterior, +/- 4.2mm medial lateral, and -5.9mm dorsoventral from the cranial surface. Injectors (33 gauge, Plastics One, VA) extended 2.5 mm from the end of the cannula to a depth of -8.4mm from the cranial surface to terminate in the central amygdala. See Figure 1 for cannulae placement.

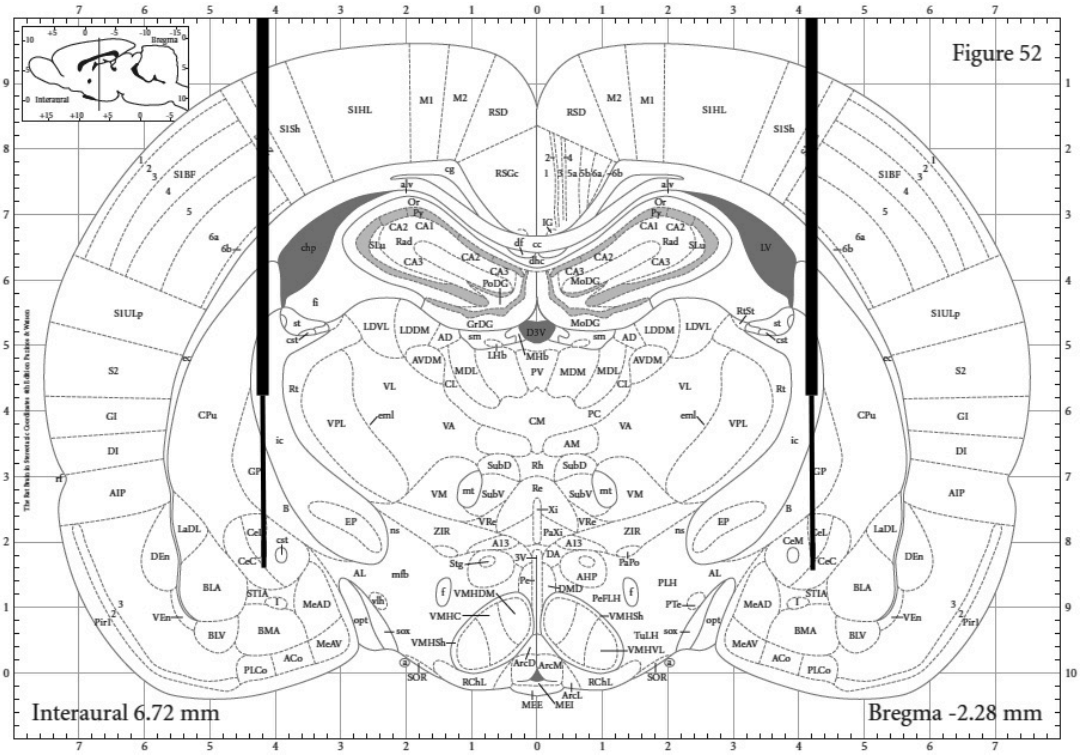

Figure 1. Schematic representation of the guide cannulae (broad bars) and injector cannulae (thin bars) targets for the CeA. The CeA image represents a coronal brain section -2.28 mm relative to bregma. The images are adapted from the atlas of Paxinos and Watson (2007).

2.3.2 Nucleus Accumbens

Guide cannulae were aimed at the following coordinates of the Paxinos and Watson Rat Brain Atlas (6th edition, 2007); relative to bregma +1.6mm anteroposterior, +/- 1.3mm medial lateral, and -5.5mm dorsoventral from the cranial surface. Injectors (33 gauge, Plastics One, VA) extended 2.5 mm from the end of the cannula to a depth of 8.0mm from the cranial surface to terminate in the nucleus accumbens shell. See Figure 2 for cannulae placement.

To show that the behavioral effects of antalarmin on naloxone-precipitated morphine withdrawal in the plus maze were a result of blocking CRF-1 receptors specifically in the targeted brain regions, guide cannulae were also aimed at a region +1mm above the NAC shell (corresponding to the NAC core) to serve as diffusion controls. These surgeries were identical to the corresponding NAC shell surgeries, except that they were placed 1mm shorter in the dorsoventral direction, relative to the cranial surface.

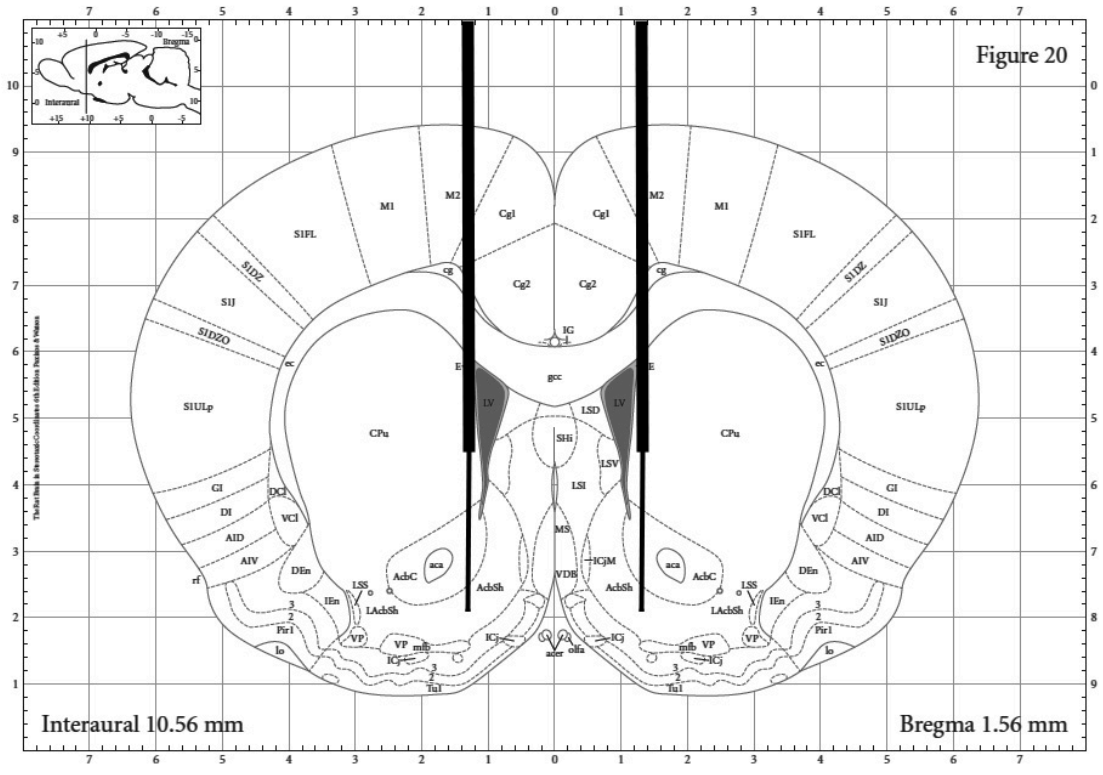

Figure 2. Schematic representation of the guide cannulae (broad bars) and injector cannulae (thin bars) targets for the NAC (shell) regions. The NAC shows a coronal slice at 1.56 mm relative to bregma. The images are adapted from the atlas of Paxinos and Watson (2007).

2.4 Blocking CRF-1 Receptors

The injectors were attached to a 10 µl Hamiliton Syringe by polyethylene tubing (Plastics One, VA) and during testing, they were filled with 10.0nmol, 3.3nmol, or 1.0nmol of antalarmin or saline (for control groups) and inserted into the guide cannulae after removing the stylets. 5 minutes before testing, 1 µl of antalarmin or saline was infused over a period of two minutes on each side using a Harvard micro-infusion pump. At the end of the infusion, the injectors stayed in an additional 60 seconds to make sure that none of the infused fluid went back into the cannulae. Stylets were placed back into the cannulae immediately following the end of the infusion.

2.5 Histological Verification

After behavioral testing was completed, permanent stylets (15mm long, extended 2.5mm past end of cannula just like the injectors used for drug infusions) were inserted into the cannulae and left for a minimum of 5 days. Animals were then euthanized using euthasol (10ml/kg) and were perfused via a transcardial perfusion with 10% formalin. The brains were placed in 10% formalin for 24 hours and were then transferred into a 30% sucrose solution for at least 48 hours. Brains were then frozen in O.C.T. Compound (Torrance, CA, USA) and cut at a thickness of 50µm in coronal slices using a cryostat sliding microtome. The sections were placed on slides and allowed to dry. The slides were stained using 0.15% cresyl violet and the permanent stylet tips visualized using the atlas of Paxinos and Watson Rat Brain Atlas as a guide. Using a series of overlaid,

coronal pictures from the Paxinos and Watson atlas, the injector tips for each animal were mapped onto the appropriate area where they were seen on the slide (See Figures 3 and 4). This was done without the knowledge of the behavioral data of each animal.

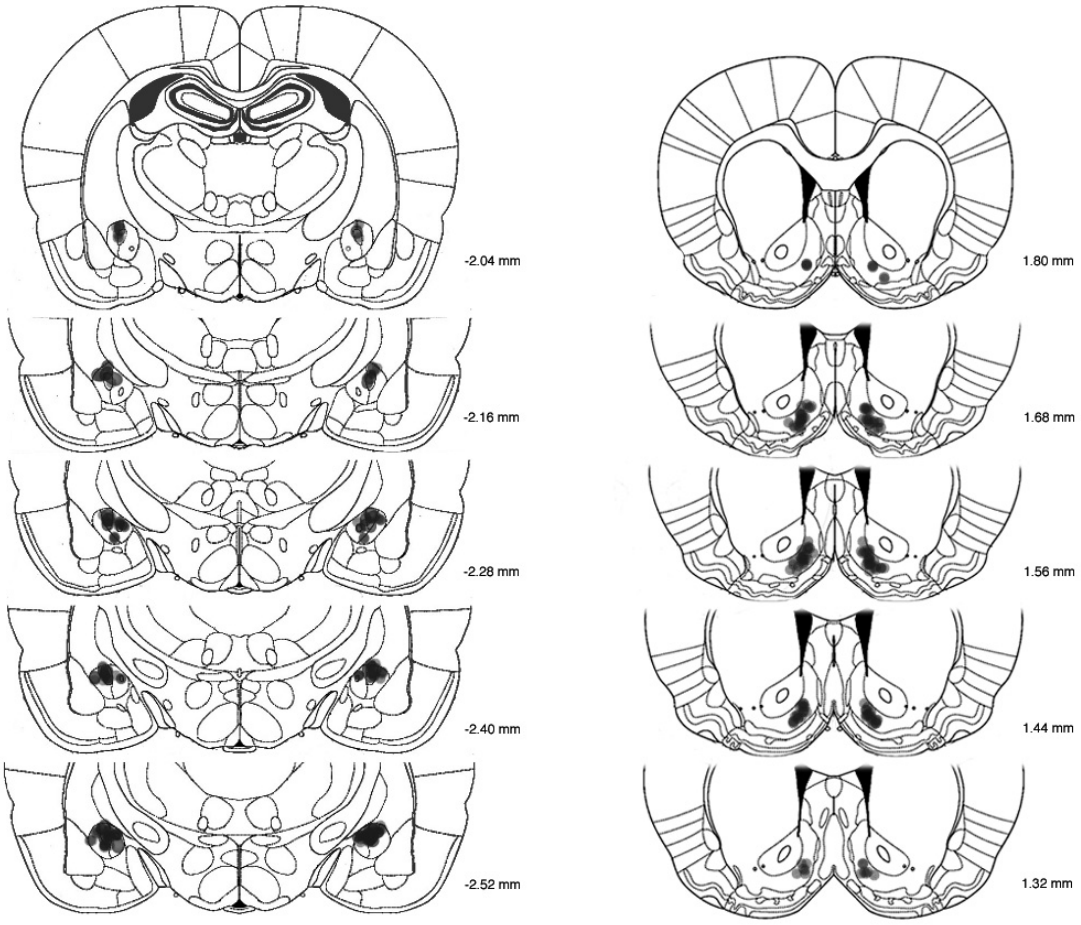

Figure 3. Injector cannula tip termination locations for the two highest doses of Antalarmin tested in the CeA and the NAC. Numbers on the side represent the AP distance from bregma. Images adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2007).

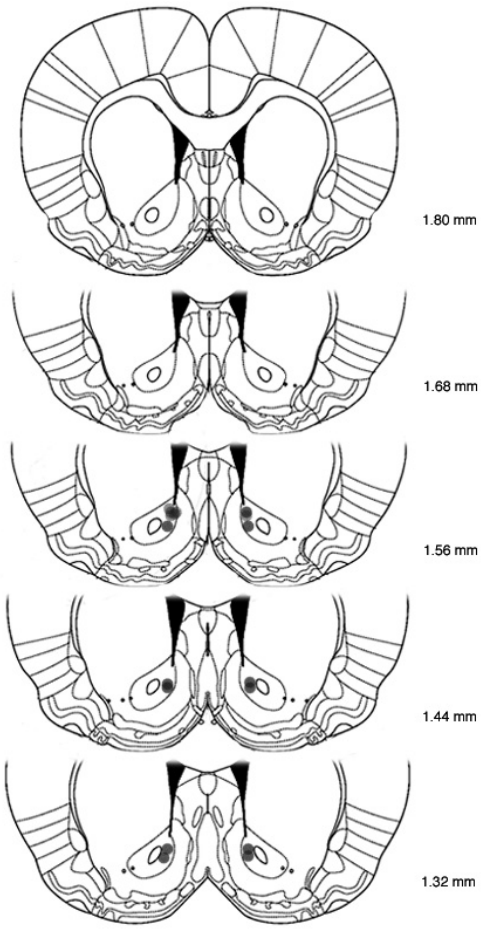

Figure 4. Injector cannula tip termination locations for diffusion control sites positioned + 1 mm above the target depth. Numbers on the side represent the AP distance from bregma. Images adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2007)

3. Effects of Blocking CRF-1 Receptors on Behavior in the Elevated Plus Maze

3.1 Apparatus

The elevated plus maze used in this experiment is the same as previously described (Zhang and Schulteis, 2008). The apparatus was acquired from Kinder Scientific (Poway, CA) and it consists of two opposing open arms, whose length is 50 cm and width is 10.8 cm. It has two opposing closed arms of equal length and width but with 33.5 cm high walls on either side except at the center of the maze. Each of the four arms is connected at a 90° angle to each other and the animal has access to any of the arms from the center of the maze. The maze is 85 cm off the floor and the position of the rats is continuously tracked by photo beam arrays embedded along the base of each arm and at the entry point to all the arms. Testing was conducted in a quiet room with approximately 65 dB of background noise provided by a white noise generator. Two 25-W light bulbs were attached to the legs of the closed arms and were positioned to direct light against the walls of the testing room. Rats were placed in the center of the maze facing one of the closed arms at the beginning of each test.

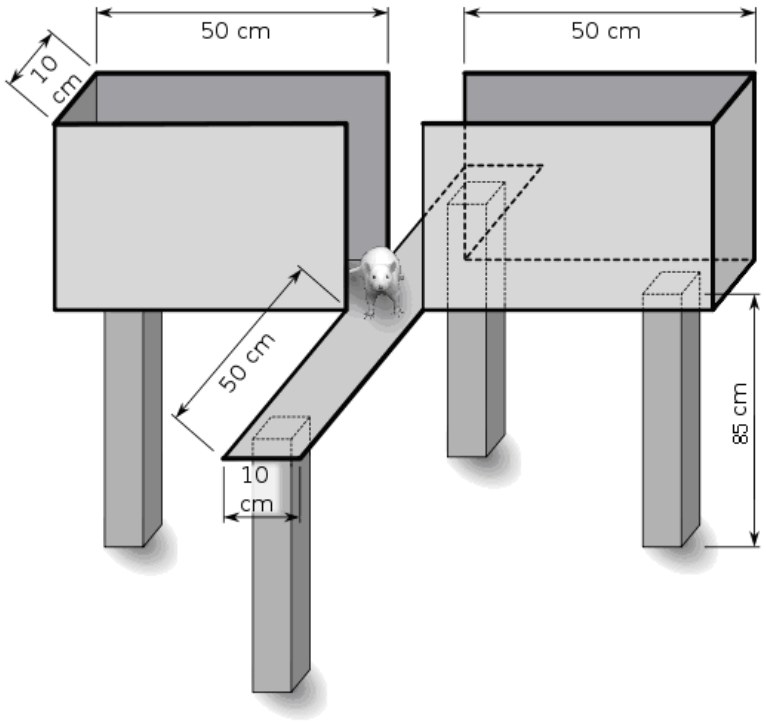

Figure 5. Schematic Drawing of the Elevated Plus Maze

3.2 Experimental Design

The experimental design used in this experiment is similar to the one previously described (Zhang and Schulteis, 2008). At least 5 days following surgery, the rats were transported to a "holding room" immediately adjacent to the plus maze testing room where they would be handled, weighed, injected, and temporarily housed before and after testing. The "testing room" was in the back behind the holding room where the rats were exposed to the maze. The lighting and background noise were the same in both the holding and testing rooms. After the initial transport into the holding room, the rats were gently handled for 5 minutes each, then weighed and injected with either morphine

(10mg/kg) or vehicle, and returned to their home cages. They became acclimated to the holding room by being kept in there for 4 hours before being returned to the colony. These procedures were repeated for 3 consecutive days. On the 4th (testing) day, the same procedures were followed but the animals were kept in the holding room instead of being returned to the colony. 8 hours following the last injection, rats were infused in the CeA or NAC (1 µl at a flow rate of 30 µl/hour) with either antalarmin or vehicle. 5 minutes following the infusion, they were injected with either naloxone or vehicle and 10 minutes following that injection, placed on the plus maze for a 5-minute test.

Table 3-1. Elevated Plus Maze Experimental Design

Day	D1	D2	D3	D4
Injection T=0h	VEH or Mor	VEH or Mor	VEH or Mor	VEH or Mor
Infusion T=7h 55min				VEH or Ant
Injection T=8h				VEH or Nal

Table 3-2. Groups Tested in Plus Maze

Morphine Condition	**Antalarmin Condition**	**Naloxone Condition**	CeA (n)	NAC (n)
Veh	***Veh***	***Veh***	***10***	***10***
Veh	***Veh***	***Nal***	***11***	***11***
Veh	Ant 3.3	Nal		11
Veh	Ant 10.0	Nal	10	
Mor	***Veh***	***Veh***	***9***	***9***
Mor	***Veh***	***Nal***	***11***	***11***
Mor	Ant 1.0	Nal		9
Mor	Ant 3.0	Nal	14	11
Mor	Ant 10.0	Nal	13	
Mor	Ant 3.3 Diffusion Control	Nal		10

3.3 Data Analysis

All data were collected by a Windows XP computer using MotorMonitor Software (Kinder Scientific, Poway, CA). The following measures were computed for each rat: 1) time spent in the open arms as a percentage of the total time spent exploring both the open and closed arms (% Time Open), 2) the number of entries in the open arms as a percentage of the total entries into both the open and closed arms (% Entries Open), and 3) the total number of entries into the closed arms (Closed Entries). The measure of general activity was Closed Entries, which is a reliable method to measure locomotor activity in the plus maze. All behavioral data are presented as an average +/- SEM. Statistical analyses consisted of appropriate one, two or three-factor ANOVAS and were

performed using Jmp Software. $P<0.05$ was set as the level of statistical significance for all statistical analyses.

4. Results

4.1 Effects of Precipitated Morphine Withdrawal on Behavior in the Plus Maze: Between Subjects Analysis

In the elevated plus-maze, anxiety-like behavior is measured by significant decreases in relative amount of time spent in the open versus closed arms of the plus maze (% Time Open), and the number of entries into the open versus closed arms (% Entries Open). The number of entries into the closed arms (# Closed Entries) is used as a validated measure of general activity. As shown in Table 4-1, for control groups receiving only Veh infusion into the CeA or NAC, a 2x2x2 ANOVA with morphine condition (Veh or Mor), naloxone condition (Veh or Naloxone), and brain region (CeA or NAC) as individual between-subjects factors revealed significant main effects of Morphine and Naloxone conditions and a significant interaction of these conditions in the % Time Open measure (Table 4-1) (Figures 6 and 9). There was no main effect of brain region, and no significant interaction of brain region with any other condition.

For % Entries Open, the main effect of naloxone and the morphine-naloxone interaction were significant; again, there was no main effect of brain region or interaction of brain region with any other factor. The significant interaction of Morphine and Naloxone Condition for both % Time Open and % Entries Open measures resulted from significant reduction in these measures when naloxone followed morphine, but not when naloxone followed vehicle, or vehicle followed morphine across both the CeA and NAC groups ($p<0.05$ vs. % Time Open and % Entries Open) (Table 4-1) (Figures 7 and 11).

There were no significant main effects or interactions found with the Closed Entries measure (Table 4-1) (Figures 8 and 12). Thus, analysis of the groups receiving Vehicle infusion into CeA or NAC revealed that naloxone-precipitated withdrawal from morphine produced a significant anxiety-like effect as measured by reduced % Open Time and % Open Entries, but the anxiety-like effects of naloxone in rats treated with morphine are not accompanied by significant changes in overall activity levels (Closed Entries) (Table 4-1).

Table 4-1. 3 Factor ANOVA of Between Subject Analysis.

Plus Maze Measure		**3 Factor ANOVA**	
% Time Open	Main Effects	Morphine Condition	(F[1,74]=9.072, P<0.005)
		Naloxone Condition	(F[1,74]=14.886, P<0.0005)
		Brain Region	(F[1,74]=1.254, N.S.)
	Interactions	Morphine-Naloxone	(F[1,74]=15.460, P<0.0005)
		Morphine-Region	(F[1,74]=0.112, N.S.)
		Naloxone-Region	(F[1,74]=2.592, N.S.)
		Morphine-Naloxone-Region	(F[1,74]=1.904, N.S.)
	Post-hoc Comparisons	Veh-Veh-Veh vs Veh-Veh-Nal	(F[1,74]=0.003, N.S.
		Veh-Veh-Nal vs Mor-Veh-Veh	(F[1,74]=0.393, N.S.)
		Veh-Veh-Nal vs Mor-Veh-Nal	(F[1,74]=26.051, P<0.0001)
		Mor-Veh-Veh vs Mor-Veh-Nal	(F[1,74]=29.508, P<0.0001)

Table 4-1. Continued.

% Entries Open	Main Effects	Morphine Condition	(F[1,74]=1.349, N.S.)
		Naloxone Condition	(F[1,74]=12.851, P<0.001)
		Brain Region	(F[1,74]=1.094, N.S.)
	Interactions	Morphine-Naloxone	(F[1,74]=7.224, P<0.01)
		Morphine-Region	(F[1,74]=0.029, N.S.)
		Naloxone-Region	(F[1,74]=1.796, N.S.)
		Morphine-Naloxone-Region	(F[1,74]=2.088, N.S.)
	Post-hoc Comparisons	Veh-Veh-Veh vs Veh-Veh-Nal	(F[1,74]=0.414, N.S.)
		Veh-Veh-Nal vs Mor-Veh-Veh	(F[1,74]=1.083, N.S.)
		Veh-Veh-Nal vs Mor-Veh-Nal	(F[1,74]=8.005, P<0.01)
		Mor-Veh-Veh vs Mor-Veh-Nal	(F[1,74]=19.131, P<0.0001)
# Closed Entries	Main Effects	Morphine Condition	(F[1,74]=2.627, N.S.)
		Naloxone Condition	(F[1,74]=0.351, N.S.)
		Brain Region	(F[1,74]=1.761, N.S.)
	Interactions	Morphine-Naloxone	(F[1,74]=0.129, N.S.)
		Morphine-Region	(F[1,74]=1.612, N.S.)
		Naloxone-Region	(F[1,74]=1.868, N.S.)
		Morphine-Naloxone-Region	(F[1,74]=0.039, N.S.)

4.2 Effects of Precipitated Morphine Withdrawal on Vehicle Controls

A One Factor ANOVA was used to analyze any differences between the vehicle controls in both the NAC and CeA experiments, comparing Veh-Veh-Veh, Veh-Veh-Nal, and Veh-Ant 3.3 (or 10.0)-Nal to each other in each brain region separately. There were no significant effects between any vehicle groups in either brain regions (see left side of Figures 6 through 12, and Table 4-2). These results were expected and validated the claim that naloxone and antalarmin do not independently reverse anxiety-like behavior in the plus maze in the absence of morphine pre-treatment (See Table 4-2).

Table 4-2. 1 Factor ANOVA of Vehicle Control Groups in NAC and CeA.

Plus Maze Measure	**Site**	**One-Factor ANOVA**	**Graph**
% Time Open	NAC CeA	(F[2,29]=2.184, N.S.) (F[2,28]=0.267, N.S.)	Figure 3-1 Figure 3-4
% Entries Open	NAC CeA	(F[2,29]=0.837, N.S.) (F[2,28]=0.699, N.S.)	Figure 3-2 Figure 3-5
# Closed Entries	NAC CeA	(F[2,29]=0.366, N.S.) (F[2,28]=0.085, N.S.)	Figure 3-3 Figure 3-6

4.3 Effects of Precipitated Morphine Withdrawal on Groups in the NAC that Received Antalarmin

A one factor ANOVA was used to determine the effect of antalarmin infusions on naloxone-precipitated withdrawal following morphine pretreatment in the NAC (each analysis included Mor-Veh-Veh, Mor-Veh-Nal, all Mor-Ant “dose x”-Nal groups, and

Diffusion Control group). There was a significant effect of treatment condition in each of the plus-maze measures ($p<0.05$ vs % Time Open, % Entries Open, and # Closed Entries) (Table 4-3). As shown in Figures 6 and 7, infusion of 3.3 nmol antalarmin into the NAC reverses the anxiety-like behavior precipitated by naloxone in morphine-treated rats, as measured by both % Time Open and % Entries Open.

For % Time Open, post-hoc comparisons revealed that Mor-Veh-Nal significantly reduced open arm time relative to Mor-Veh-Veh as expected. The naloxone-induced reduction in % Time Open was significantly attenuated in the Mor-Ant 3.3-Nal but not Mor-Ant 1.0-Nal groups; however, Mor-Ant 3.3-Nal also remained significantly different from Mor-Veh-Veh, indicating an incomplete reversal of the anxiety-like effect. The effect of antalarmin 3.3 was absent when infused into the diffusion control site (this group did not differ from Mor-Veh-Veh), indicating that the effects observed in the NAC shell were site-specific and not the result of diffusion up the cannula tract into the NAC core (Table 4-3) (Figure 6).

For % Entries Open, the Mor-Veh-Nal group significantly reduced the percentage of entries relative to Mor-Veh-Veh as predicted. The naloxone-induced reduction in % Entries Open was significantly attenuated in the Mor-Ant 3.3-Nal but not Mor-Ant 1.0-Nal groups, as it was in the % Time Open. The Mor-Ant 3.3-Nal group, however, was significantly different from Mor-Veh-Veh, which signified a complete reversal of the anxiety-like effect in the % Entries measure. The effect of antalarmin 3.3 was again absent when infused into the diffusion control site and this group did not differ from Mor-Veh-Veh (Table 4-3) (Figure 7).

For the activity measure (Closed Entries), the Mor-Ant 3.3-Nal group was significantly different from both the Mor-Veh-Veh and Diffusion Control groups, with less closed entries by the groups infused with antalarmin 3.3 into the NAC shell (Table 4-3) (Figure 8).

Table 4-3. 1 factor ANOVA of NAC Groups that Received Antalarmin and Morphine.

Plus Maze Measure	**Post-hoc Comparisons**	**One-Factor ANOVA**
% Time Open	**Treatment Condition**	**(F[4,44]=16.340, P<0.0001)**
	Mor-Veh-Veh vs. Mor-Veh-Nal	(F[1,44]=45.578, P<0.0001)
	Mor-Veh-Veh vs. Mor-Ant 1.0-Nal	(F[1,44]=26.333, P<0.0001)
	Mor-Veh-Veh vs. Mor-Ant 3.3-Nal	(F[1,44]=7.735, P<0.01)
	Mor-Veh-Nal vs. Mor-Ant 1.0-Nal	(F[1,44]=1.879, N.S.)
	Mor-Veh-Nal vs. Mor-Ant 3.3-Nal	(F[1,44]=16.162, P<0.0005)
	Mor-Veh-Nal vs. Diffusion Control	(F[1,44]=0.000, N.S.)
	Mor-Ant 3.3-Nal vs Diffusion Control	(F[1,44]=15.509, P<0.0005)
% Entries Open	**Treatment Condition**	**(F[4,44]=9.229, P<0.0001)**
	Mor-Veh-Veh vs. Mor-Veh-Nal	(F[1,44]=19.987, P<0.0001)
	Mor-Veh-Veh vs. Mor-Ant 1.0-Nal	(F[1,44]=6.870, P<0.01)
	Mor-Veh-Veh vs. Mor-Ant 3.3-Nal	(F[1,44]=0.244, N.S.)
	Mor-Veh-Nal vs. Mor-Ant 1.0-Nal	(F[1,44]=2.964, N.S.)
	Mor-Veh-Nal vs. Mor-Ant 3.3-Nal	(F[1,44]=16.646, P<0.0005)
	Mor-Veh-Nal vs. Diffusion Control	(F[1,44]=0.014, N.S.)
	Mor-Ant 3.3-Nal vs Diffusion Control	(F[1,44]=16.823 P<0.0005)
# Closed Entries	**Treatment Condition**	**(F[4,44]=2.797, P<0.05)**
	Mor-Veh-Veh vs. Mor-Veh-Nal	(F[1,44]=1.635, N.S.)
	Mor-Veh-Veh vs. Mor-Ant 1.0-Nal	(F[1,44]=0.179, N.S.)
	Mor-Veh-Veh vs. Mor-Ant 3.3-Nal	(F[1,44]=9.289, P<0.005)
	Mor-Veh-Nal vs. Mor-Ant 1.0-Nal	(F[1,44]=0.698, N.S.)
	Mor-Veh-Nal vs. Mor-Ant 3.3-Nal	(F[1,44]=3.570, N.S.)
	Mor-Veh-Nal vs. Diffusion Control	(F[1,44]=0.097, N.S.)
	Mor-Ant 3.3-Nal vs Diffusion Control	(F[1,44]=4.627, P<0.05)

4.4 Effects of Precipitated Morphine Withdrawal on Groups that Received Antalarmin in the CeA

A one factor ANOVA was used to determine the effects of antalarmin infused into the CeA on naloxone-precipitated morphine withdrawal effects in the plus maze. A main effect of antalarmin condition was found to be significant in only the % Time Open plus maze measure ($p<0.05$) (Table 4-4). Post-hoc comparisons for each of the morphine conditions indicated that there was a significant difference in % Time Open between morphine-vehicle and morphine-naloxone groups. Neither antalarmin 3.3 nmol nor antalarmin 10 nmol reversed the decrease in % time spent in the open arms of the plus maze ($p<0.05$ vs % Time Open) (Figure 9). Thus, antalarmin infused into the CeA did not attenuate the decrease in open arm time as it did when infused into the NAC. There was also not a change in the activity level between the groups, as indicated by the number of closed arm entries (Figure 12).

Table 4-4. 1 factor ANOVA of CeA Groups that Received Antalarmin and Morphine.

Plus Maze Measure	**Post-hoc Comparisons**	**One-Factor ANOVA**
% Time Open	**Antalarmin Condition**	**(F[3,43]=5.067, P<0.005)**
	Mor-Veh-Veh vs. Mor-Veh-Nal	(F[1,43]=11.710, P<0.001)
	Mor-Veh-Veh vs. Mor-Ant 3.3-Nal	(F[1,43]=12.001, P<0.001)
	Mor-Veh-Veh vs. Mor-Ant 10.0-Nal	(F[1,43]=8.243, P<0.01)
	Mor-Veh-Nal vs. Mor-Ant 3.3-Nal	(F[1,43]=0.021, N.S.)
	Mor-Veh-Nal vs. Mor-Ant 10.0-Nal	(F[1,43]=0.512, N.S.)
% Entries Open	**Antalarmin Condition**	**(F[3,43]=2.034, N.S.)**
# Closed Entries	**Antalarmin Condition**	**(F[3,43]=0.286, N.S.)**

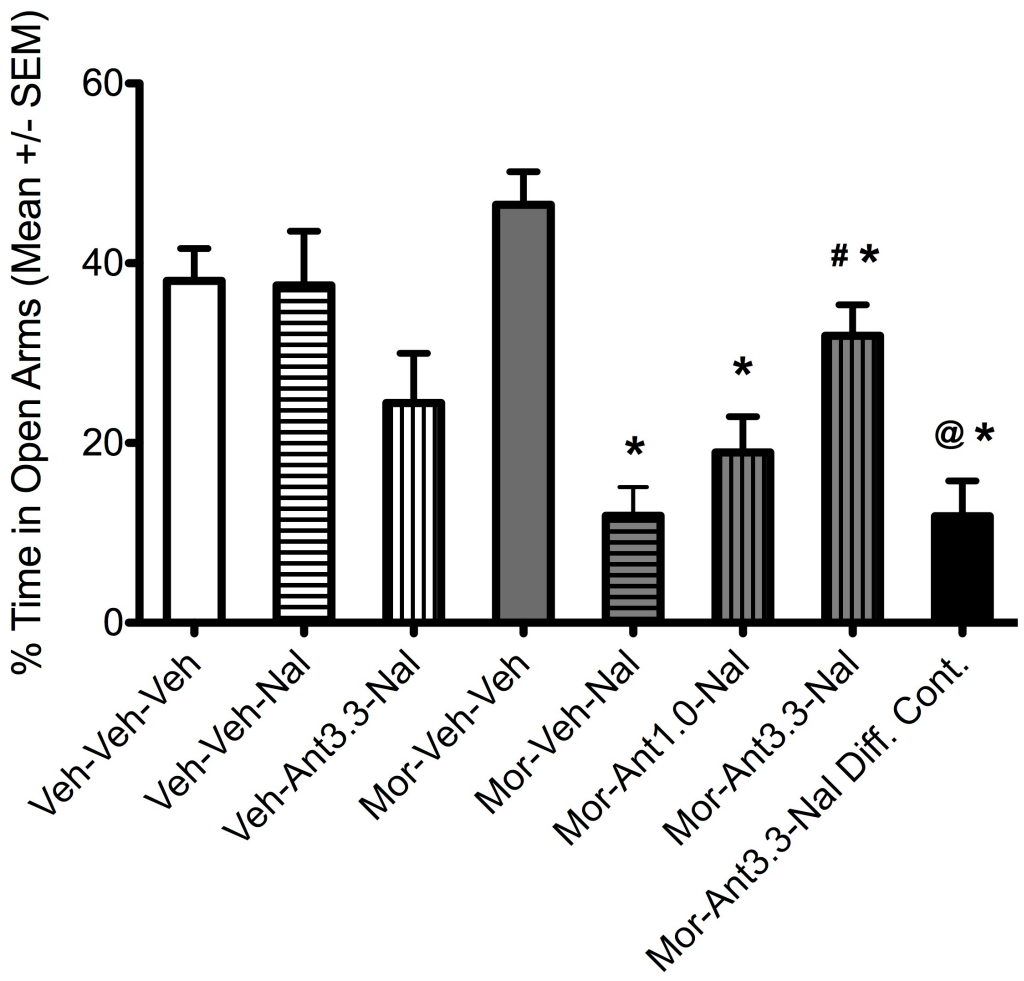

Figure 6. % Time Open in NAC. The time spent in open arms as a percentage of the total time spent in open and closed arms in the NAC. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The diffusion control group is highlighted in black. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. The * symbolizes groups that are significantly different from Mor-Veh-Veh. The # symbolizes groups that are significantly different from the Mor-Veh-Nal group. The @ symbolizes groups that are different from Mor-Veh-Veh and Mor-Ant 3.3-Nal. Data represents percent time spent in open arms (± S.E.M).

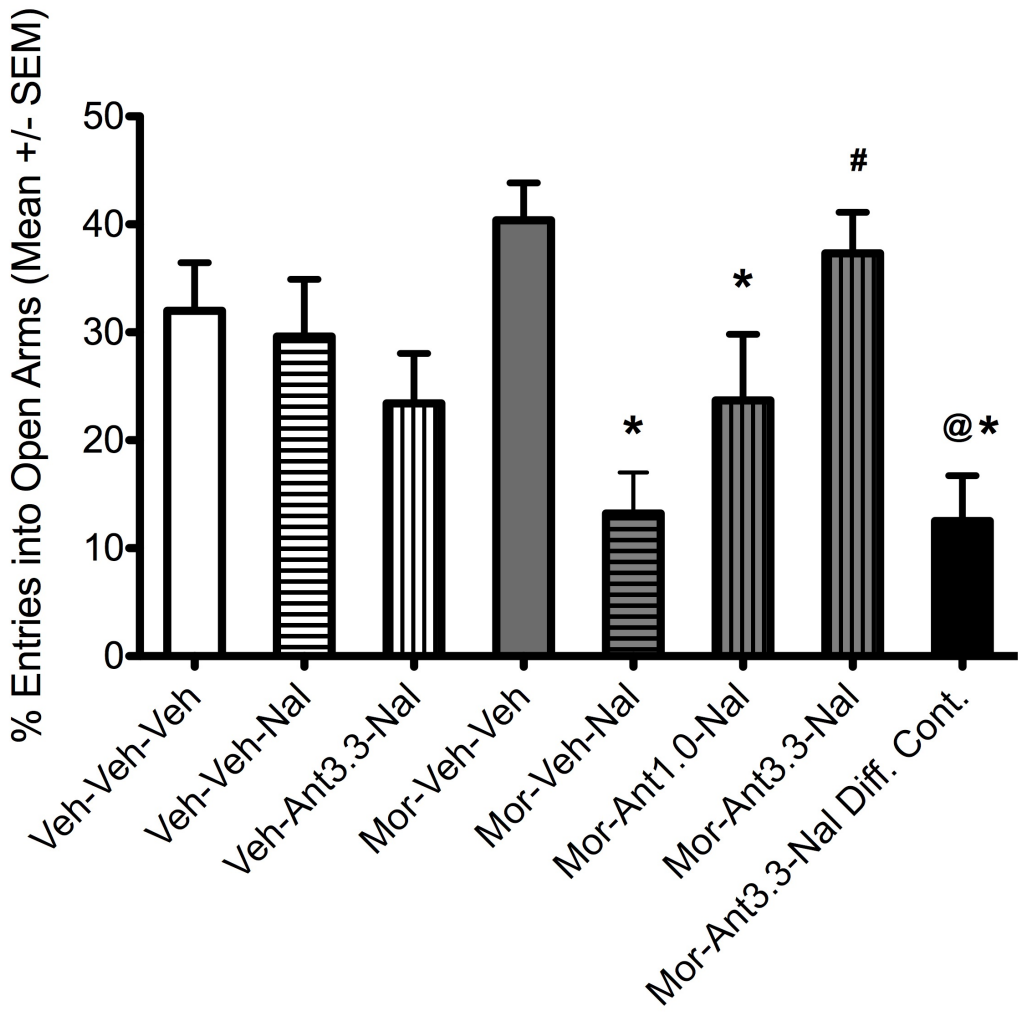

Figure 7. % Entries Open in NAC. The number of entries into the open arms calculated as a percentage of the total number of entries into the open and closed arms in the NAC. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The diffusion control group is highlighted in black. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. The * symbolizes groups that are significantly different from Mor-Veh-Veh. The # symbolizes groups that are significantly different from the Mor-Veh-Nal group. The @ symbolizes groups that are different from Mor-Veh-Veh and Mor-Ant 3.3-Nal. Data represents percent number of entries made into the open arms (± S.E.M).

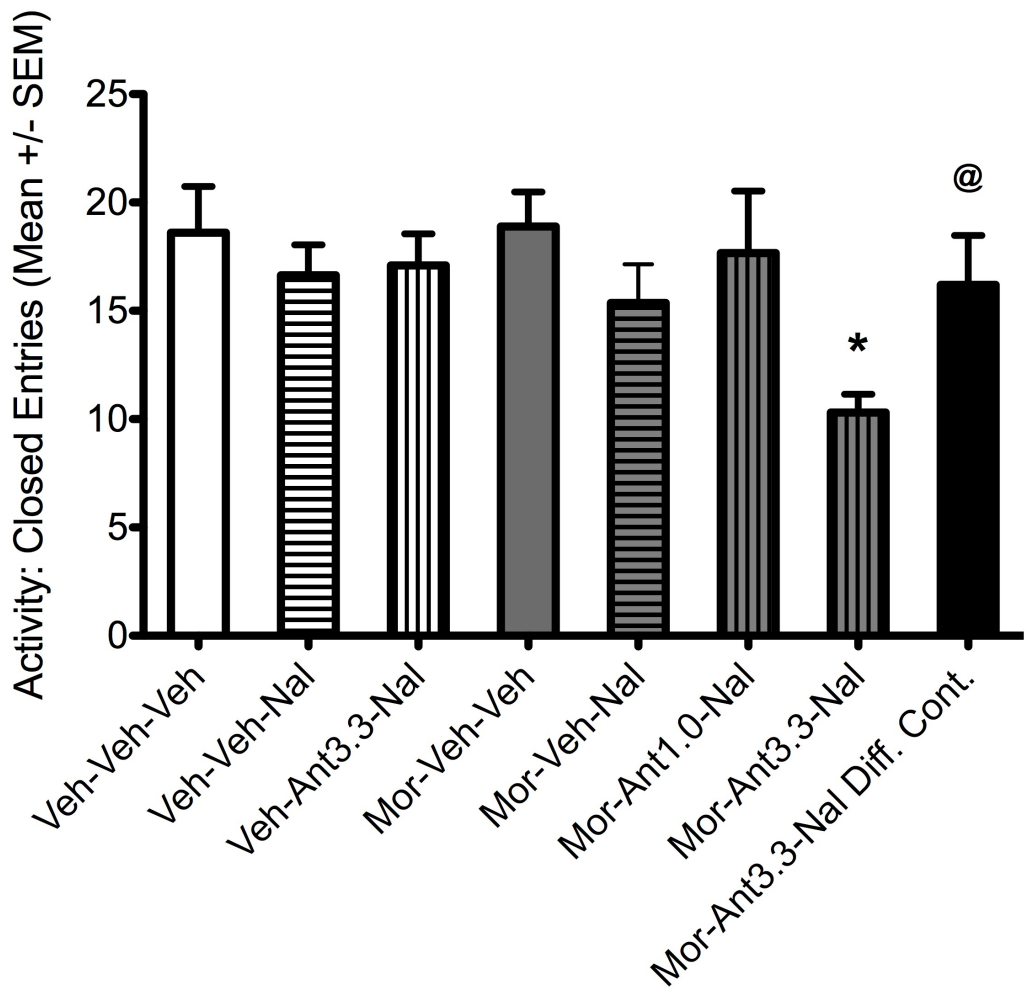

Figure 8. # Closed Entries in NAC. The number of entries made into the closed arms in the NAC. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The diffusion control group is highlighted in black. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. The * symbolizes groups that are significantly different from Mor-Veh-Veh. The @ symbolizes groups that are different from Mor-Ant 3.3-Nal. Data represents the number of entries made into the closed arms (± S.E.M).

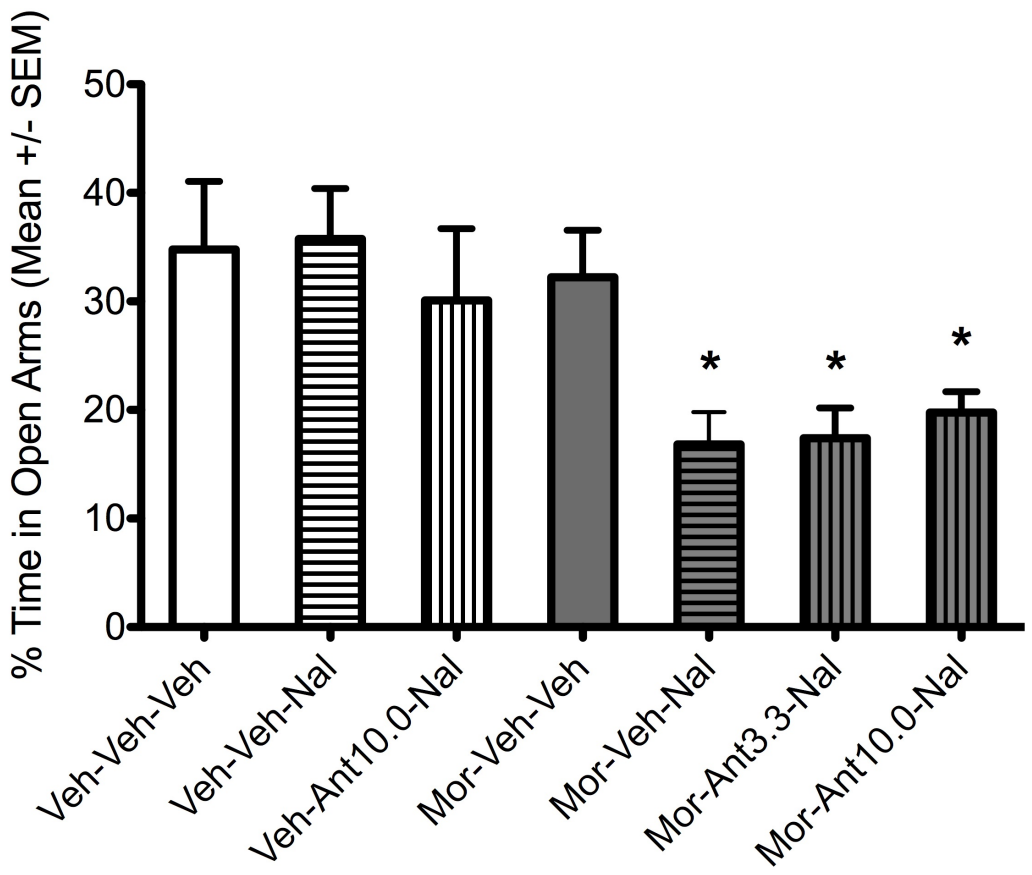

Figure 9. % Time Open in CeA. The time spent in open arms as a percentage of the total time spent in open and closed arms in the CeA. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. The * symbolizes groups that are significantly different from Mor-Veh-Veh. Data represents percent time spent in open arms (± S.E.M).

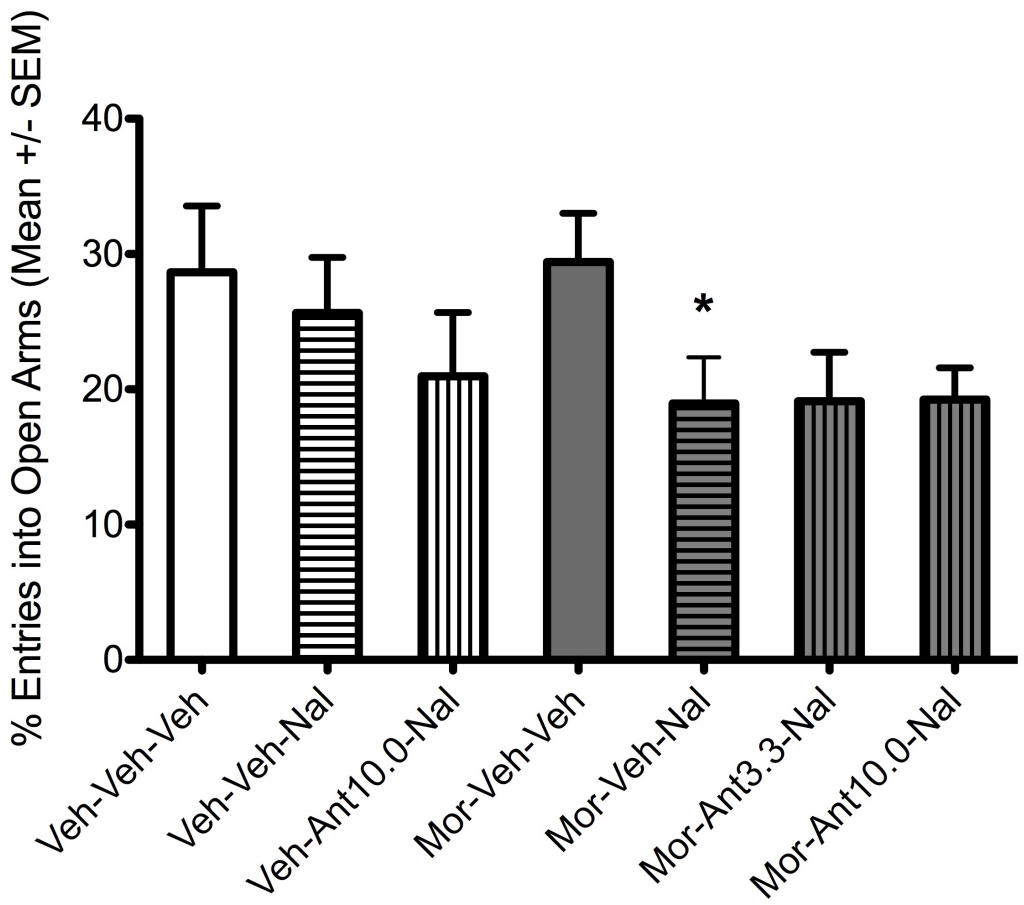

Figure 10. % Entries Open in CeA. The number of entries into the open arms calculated as a percentage of the total number of entries into the open and closed arms in the CeA. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. The * symbolizes groups that are significantly different from Mor-Veh-Veh. Data represents percentage of entries into open arms (± S.E.M).

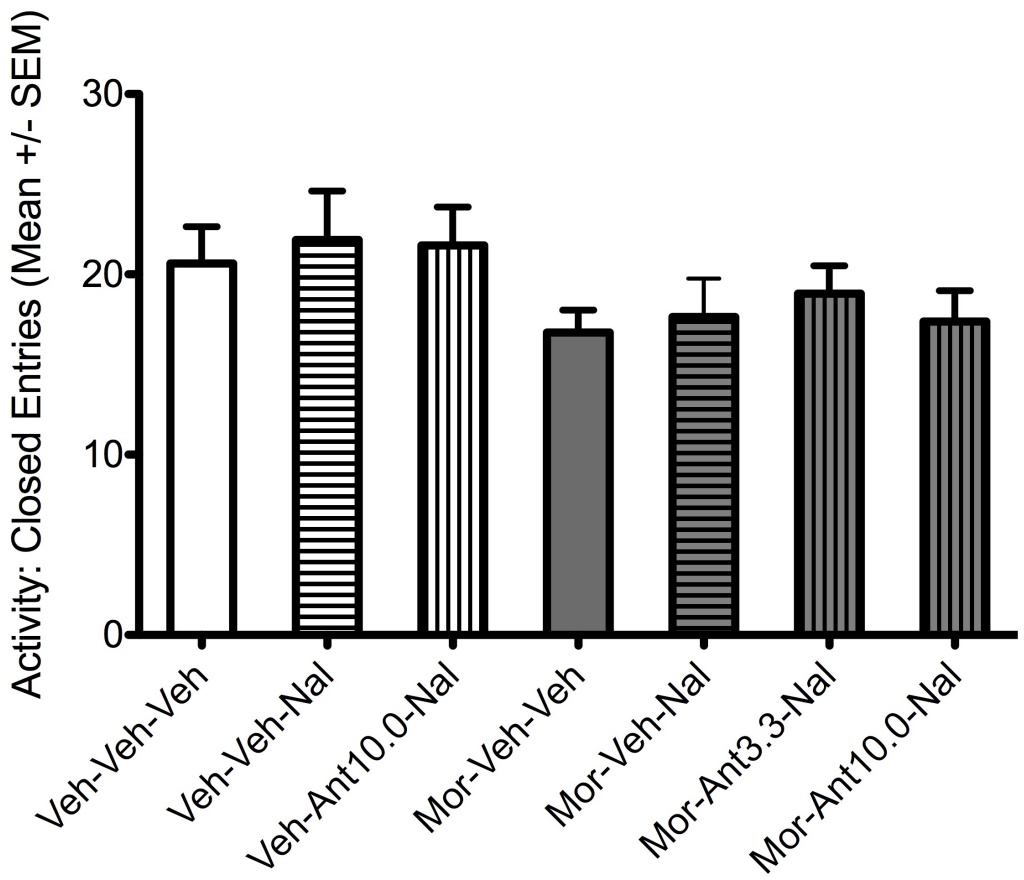

Figure 11. # Closed Entries in CeA. The number of entries made into the closed arms in the CeA. Groups that received Veh are highlighted in white and groups that received Mor are highlighted in gray. The horizontal stripes signify groups that received Veh and Nal and the vertical stripes signify groups that received Ant and Nal. Data represents the number of entries made into the closed arms (± S.E.M).

5. Discussion

This project evaluated the role of CRF-1 receptors in the extended amygdala in mediating the anxiety-like behavior produced by morphine withdrawal, by reversibly blocking the CRF-1 receptors in the extended amygdala and measuring those changes in plus maze behavior. The plus maze behaviors that were used in analysis were the percentage of time that was spent in the open arms of the plus maze (% Time Open), the percentage of entries made into the open arms (% Entries Open), and the number of entries made into the closed arms (# Closed Entries). It was found that antalarmin attenuated morphine withdrawal-induced anxiety in the shell of the NAC, as shown by a reversal of the naloxone-induced decrease in relative time spent, and number of entries into, the open arms versus the closed arms of the plus maze. Antalarmin infused into the CeA, however, was ineffective at reversing the effects of naloxone-precipitated morphine withdrawal in the plus maze. Thus, antalarmin in the NAC shell, but not core, was able to effectively attenuate the increased anxiety-like behavior in rats produced by precipitated withdrawal from morphine.

The antalarmin condition had a dose-dependent, statistically significant effect across all three of the plus maze measures when infused into the shell of the NAC. It effectively attenuated the anxiety-like behavior that the rats exhibited when given morphine and naloxone together, as measured by % of time spent and % of entries into the open arms of the maze (at 3.3 nmol but not 1.0 nmol). The effects of antalarmin on withdrawal-induced anxiety in the plus maze were specific to the NAC shell since antalarmin infused into the diffusion control site 1 mm above the NAC shell

(corresponding to the NAC core) did not attenuate the morphine withdrawal effect on any of the plus maze measures (Figures 6, 7, 8). However, antalarmin 3.3 nmol infused into the NAC shell (but not core) in animals treated with morphine and naloxone significantly reduced activity in the plus maze as measured by the number of closed entries (Figure 8); this activity effect was not evident in rats that received antalarmin 3.3 and naloxone in the absence of morphine pretreatment.

CRF has been show to have a stimulating effect on activity as part of its arousing effects (Heinrichs and Joppa, 2001). The attenuation by infusion of antalarmin into the NAC of the anxiety-like behavior could be linked to the arousal effect of the CRF system stimulation during precipitated opioid withdrawal, with antalarmin dampening the arousal effect. It has been previously shown that antalarmin decreases CRF-1 receptor-induced increases in locomotor activity (Zorrilla et al., 2002). This is consistent with the fact that stimulant drugs like amphetamine and cocaine exert their locomotor-stimulating effects via the dopamine projections from the ventral tegmental area to the NAC, and that these stimulant drugs appear to produce arousal-mediated anxiety-like behavior in rats at the same doses that stimulate locomotor activity and produce rewarding effects as measured by self-administration of the stimulant (e.g. Ettenberg, 2009). For example Ettenberg and Geist (1991) have used an operant runway as a tool to study goal-seeking motivated behavior. The animals ran down a runway to the goal box at the end; once in the chamber at the end of the runway, the rats pressed a lever to receive an intravenous infusion of cocaine. Over repeated trials, while the animals would invariably enter the goal box ultimately, they began taking progressively longer to reach the goal box. They exhibited a unique stop and retreat behavior as the experiment advanced; they would approach the

goal box, stop, and retreat back to the start box. It seemed that the animals were in conflict whether to enter the goal box and that cocaine use could exhibit both positive and negative associations with the box. Manipulations of CRF system modulate the strength of the observed "goal box avoidance" in animals running in a straight alley for intravenous cocaine. These studies suggest that cocaine administration in animals can produce anxiogenic consequences in addition to its euphoric effects (Ettenberg, 2009; Heinrichs et al., 1998). Therefore, it is possible that antagonism of CRF in the NAC shell may blunt the arousal/stimulating effects of excess CRF during morphine withdrawal and thereby reduce the anxiety-like effect.

Unlike the NAC, CRF antagonism in the CeA did not have an effect on morphine withdrawal-induced anxiety behavior in the plus maze. Specifically, the infusion of antalarmin into the CeA did not block the anxiety-like behavior in the plus maze as infusion into the NAC shell did, even at a higher concentration of antalarmin (10.0nmol) than was found effective in the NAC (3.3 nmol). This stands somewhat in contrast to other studies in which intra-amygdala administration of a CRF antagonist reversed the decrease in exploration of the open arms of a plus maze caused by ethanol withdrawal (Menzaghi et al., 1994; Rassnick et al., 1993).

Moreover, the CeA has been shown in other studies to be an important extrahypothalamic site at which CRF antagonists could effectively block conditioned opioid withdrawal effects, in paradigms where unique contextual cues are paired with naloxone-precipitated withdrawal, and ultimately the paired cues will elicit withdrawal signs similar to those produced by naloxone, such as avoidance of the naloxone-paired environment, or suppression of operant responding for food reward (Heinrichs et al.,

1995) . Thus, it is possible that the CeA may participate in conditioned withdrawal from morphine, but may not mediate directly the negative emotional signs of withdrawal such as anxiety.

However, another study in the Schulteis laboratory has shown that antalarmin infused into the CeA as well as the NAC shell decreased the dysphoria-like effects that result from precipitated opioid withdrawal, as measured in a brain stimulation reward paradigm (Chang, Schulteis, unpublished observations). Thus although the current studies indicate that the CeA is not a critical site for CRF-1 receptor effects on withdrawal-induced anxiety as measured in the plus maze, the CeA may be an important site of CRF-1 receptor effects on other withdrawal-induced negative affective symptoms.

Based on the results of the study, the NAC should be studied further as a possible substrate of the anxiety-like consequences of opioid withdrawal, and perhaps withdrawal from other drugs of abuse as well. In addition, future studies could assess the role of CRF in negative emotional signs of withdrawal in other brain areas shown to be important in drug dependence and anxiety such as the basolateral amygdala (BLA) and bed nucleus of the stria terminalis (BNST), which are both rich in CRF receptors. Further work in these areas may reveal other brain regions that are an important link between the CRF system and anxiety.

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