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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Antiadrenergic Agents Infused into the Central Amygdala Attenuate Brain
Reward Deficits during Acute Opioid Withdrawal in Rats**

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

in

Biology

by

Ravi Wettasinghe

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Professor Stefan Leutgeb, Co-Chair
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2014

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2014

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List of Abbreviations

aCSF	Artificial Cerebrospinal Fluid
BNST	Bed Nucleus of the Stria Terminalis
BSR	Brain Stimulation Reward
CeA	Central Amygdala
CRF	Corticotrophin Releasing Factor
ICSS	Intra cranial Self-Stimulation
Mor	Morphine
Nal	Naloxone
NE	Norepinephrine
Praz	Prazosin
SC	Subcutaneous
UK	UK 14,304
Veh	Vehicle

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ABSTRACT OF THE THESIS

**Antiadrenergic Agents Infused into the Central Amygdala Attenuate Brain
Reward Deficits during Acute Opioid Withdrawal in Rats**

by

Ravi Wettasinghe

Master of Science in Biology

University of California, San Diego, 2014

Professor Gerhard Schulteis, Chair
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Drug addiction is a chronic relapsing disorder characterized by withdrawal, loss of control and high rates of relapse. Avoidance of the aversive consequences of withdrawal can progressively become an increasingly important motivating factor for addicted individuals to continue their destructive habit. The motivational consequences of withdrawal may begin after a single, acute, dose of an addictive drug, which causes rapid neuroadaptation in brain stress circuitry (Schulteis 2010). Of particular interest in the study of withdrawal are the stress neurotransmitters Norepinephrine (NE) and Corticotrophin Releasing Factor

(CRF). The present study examined the neuro-adaptive changes in NE signaling that follow a single bout of morphine intoxication in rats. Using the intracranial self-stimulation (ICSS) paradigm to measure brain stimulation reward (BSR) thresholds, the role of NE in the central amygdala (CeA) was examined during acute withdrawal. Antagonism of $\alpha 1$ adrenoreceptors in the CeA with prazosin dose- dependently attenuated the depression-like increases in BSR thresholds produced by precipitated acute morphine withdrawal with the opioid antagonist naloxone. Reduction in NE release with the $\alpha 2$ autoreceptor agonist UK 14,304 did not significantly decrease thresholds. NE activity in the amygdala, in conjunction with CRF, may contribute in part to the dysphoria-like state of opioid withdrawal. These results provide further evidence for a CRF/NE feed-forward mechanism which might amplify stress responses in the development of opioid dependence. Pharmacological manipulation of NE and CRF systems can potentially lead to therapeutic treatment of opioid addiction through reducing the aversive affective states produced by withdrawal-induced hyperactivity of these stress systems.

1. Introduction

Drug abuse, addiction and overdose have enormous costs to society in terms of human suffering, legal issues, and healthcare costs. Opioids have long been used and abused for their ability to mimic natural endorphins, producing states of euphoria and analgesia. The reinforcing euphoric effects make opioids some of the most addictive substances known to man, and rates of prescription opioid abuse have risen in recent years (Sehgal et al., 2012). The age adjusted prescription opioid related death rates for 15-64 year olds increased approximately four-fold between 1999 to 2009 (Calcaterra et al., 2013).

Withdrawal from use of opioids such as morphine, heroin, Vicodin and Oxycontin include flu like symptoms, hyperalgesia, anxiety, dysphoria/depression, diarrhea, increased heart rate, and restlessness. Rapid neuroadaptation results in symptoms of withdrawal during drug abstinence, as the brain progressively adapts to disruptions in normal function due to the neuroactive agents. Over time, the motivation for ongoing substance use may transition from impulsive (positive reinforcement, e.g. seeking pleasure) to compulsive (negative reinforcement, e.g. avoiding negative affective withdrawal signs) drug use as one becomes addicted (Koob 2001). Elucidating the neural correlates in this transition can provide information on how to treat and manage opioid addiction.

The severity of affective withdrawal symptoms is often enough to motivate self-medication with continued opioid use in dependent individuals, as they

transition through cycles of drug use, withdrawal, craving, and more drug use (Koob and Le Moal 2001). Early studies on the effects of opioids have shown that symptoms of withdrawal can be elicited after a single, acute, dose of opioid, a phenomenon named “acute dependence” (Martin and Eades 1964). It has been demonstrated that negative emotional states may be more sensitive than somatic (physical, e.g. diarrhea, vomiting) withdrawal signs during acute withdrawal, highlighting the rapid neuroadaptation that results from a single bout of opioid intoxication (Schulteis 2010).

Recent advances in neuroscience have linked specific brain regions to the modulation of specific behaviors, emotions, and sensory perceptions. The rewarding properties of opioids have been linked to the activation of mu opioid receptors in the Ventral Tegmental Area and Nucleus Accumbens (Koob and Le Moal, 2001). These rewarding areas are in turn connected to limbic system structures such as the amygdala, which regulate other affective states. The amygdala has long been shown to modulate fear, arousal, learning and memory (Roosendaal et al., 2012). Lesion studies have shown the Central Amygdala (CeA) to be a key component in creating the aversive consequences of morphine and ethanol withdrawal (Moller et al., 1997; Watanabe et al., 2002). However, the CeA does not stand alone in this regard, and a group of several brain regions including the bed nucleus of the stria terminalis (BNST) and shell of the nucleus accumbens (NAcc) alongside the CeA have been shown to modulate reward and stress responses to addictive drugs. This group of interconnected structures, the

so- called “extended amygdala,” has been revealed to undergo the rapid neuroadaptation characteristic of acute dependence (Koob 2003), even after a single bout of morphine intoxication (Criner et al., 2007).

Particular acute affective symptoms of withdrawal, such as anxiety, anhedonia, and dysphoria, can be studied in animal models. Anhedonia is a reward deficit disorder in which normally pleasurable stimuli fail to elicit pleasure. Dysphoria, opposite to euphoria, describes a state of malaise that is often experienced in conjunction with anhedonia. Intracranial Self-Stimulation (ICSS) is an operant paradigm where rodents can self-administer electrical stimulation to reward circuitry. Stimulation of brain reward pathways has been described as the single unifying component of all highly addictive drugs including cocaine, other psychostimulants (e.g. amphetamines, nicotine), and opioids, among others (Kornetsky 1979). The ICSS paradigm is particularly useful in examination of the euphoric or dysphoric effects of substances in the study of drug addiction (Schulteis, 2010). Rats have elevated brain stimulation reward (BSR) thresholds during drug withdrawal (dysphoria-like), and decreased thresholds while intoxicated with reinforcing psychoactive drugs (euphoria-like). ICSS is able to quantitatively measure deficits or increases in reward function through these changes in BSR thresholds (Markou and Koob, 1992).

Rats have a well characterized central nervous system with neurochemical pathways that correlate with humans, allowing translational research. The infusion of neurotransmitter analogs or antagonists into discrete

brain regions can be used to gauge the behavioral/affective properties of differing neurochemical systems. The rapid elicitation of withdrawal signs following administration of naloxone (or other opioid antagonists) in a rat intoxicated by morphine (or other opioid agonists) is termed precipitating withdrawal. Previous studies in the Schulteis lab have used ICSS in combination with a discrete micro-injection paradigm to study the effects of CRF-1 receptor antagonists in the CeA on acute naloxone precipitated morphine withdrawal (Chang and Schulteis 2011). The study found that the CRF-R1 antagonist antalarmin, when infused into the CeA of the rat brain, reversed the dysphoric effects seen in ICSS during acute naloxone precipitated morphine withdrawal.

It has been shown that CRF systems interact closely with norepinephrine (NE) systems in activating of brain stress systems, possibly through a feed-forward mechanism (Koob 1999). NE can stimulate the release of CRF to activate the hypothalamic-pituitary-adrenal axis (Dunn et al., 2004). A study on hypoxia-induced release of CRF in rats found that NE antagonists could reverse this hypoxia-induced CRF release in the hypothalamus. The same study found the NE-stimulating drug yohimbine could stimulate CRF release in the rat hypothalamus (Chen et al., 2004). Another study found that prazosin and clonidine, two drugs which reduce NE action, blocked CRF agonist induced startle responses in rats (Gresack and Risbrough 2011). The same study showed that the α_2 noradrenergic antagonist atipamezole increased startle by increasing NE, and this was partially attenuated by CRF antagonist pretreatment. These

results point toward reciprocal interactions between NE and CRF systems to modulate stress responses. A feed-forward mechanism can theoretically account for over activated stress responses in those with anxiety disorders, but could also contribute to the progressive development of stress system hyperactivity resulting from the development of drug dependence (Koob 1999).

Dense NE innervation has been identified in regions of the extended amygdala. NE signaling from the nucleus tractus solarius and locus coeruleus have been implicated in activation of these limbic structures during opioid withdrawal (Smith and Aston-Jones, 2008). Studies using drugs to reduce NE have shown positive results in attenuation of withdrawal signs associated with nicotine, cocaine, morphine, and heroin dependence (Newton et al., 2012; Yamada and Bruinjeel, 2010; Powell et al., 2011). A clinical trial demonstrated that the α_1 receptor antagonist doxazosin diminished desires to use cocaine in cocaine dependent volunteers (Newton et al., 2012). Yamada and Bruinjeel found that stimulation of α_2 -adrenergic receptors, which act to reduce NE release through presynaptic autoreceptors, in the CeA attenuates stress-induced reinstatement of nicotine seeking in rats (Yamada and Bruinjeel, 2010). Powell and colleagues (2011) found that systemic administration of the α_2 -adrenergic agonists clonidine and UK 14,304 were more effective in reversing BSR threshold elevations during precipitated withdrawal from repeat than from acute morphine treatment. BSR threshold elevations during withdrawal from acute morphine intoxication were reversed, in contrast, by the CRF antagonist MPZP.

However, both clonidine and UK 14,304 more completely reversed anxiety-like behavior exhibited in the elevated plus maze paradigm during withdrawal from both acute and repeat morphine intoxication, whereas CRF antagonists were much less effective (Powell et al., 2011). These results suggest a primary role for NE in mediating the anxiety-like consequences of withdrawal from acute morphine, but that CRF may play the predominant initial role in expression of dysphoria-like consequences, with NE progressively recruited through the feed-forward loop upon repeated opioid experience.

This thesis focuses on the role of NE on withdrawal from a single acute bolus dose of morphine in a well-characterized model of acute opioid dependence (e.g. Liu and Schulteis, 2004). As alluded to in preceding paragraphs, several receptor types in the brain respond to NE. The α_1 receptor is located primarily on postsynaptic terminals and can be blocked by antagonists such as prazosin; α_1 receptors are primarily Gq-coupled receptors that ultimately act to depolarize cells. The α_2 receptor lies primarily on presynaptic NE axon terminals as an auto-receptor; α_2 receptors are primarily Gi receptors that act to hyperpolarize the cell. Therefore stimulation causes a reduction of NE release, and α_2 agonists are used to achieve a reduction of NE in the synapse (Yamada and Bruinjeel 2011). Clonidine is an α_2 agonist that has long been used to treat the somatic symptoms of opioid withdrawal during abstinence (Gold et al., 1978).

The highly specific α_2 agonist UK 14,304 and the α_1 antagonist prazosin were used for this thesis project. To investigate their role in the CeA on opioids

withdrawal, these antiadrenergics were infused into the CeA just before naloxone administration, in rats given morphine 4 hours earlier. Rats were then put through regular behavioral ICSS testing to observe quantifiable changes in their BSR thresholds as a measure of affective state. The antiadrenergic agents were shown to contribute to a partial attenuation of threshold elevations, providing evidence for a partial role for NE in the CeA in the dysphoria-like symptoms of opioid withdrawal.

2. Materials and Methods

2.1 Animals

Male Wistar rats (n=105) from Harlan Sprague Dawley (Livermore, CA) weighing 200-225 grams upon arrival were used. Animals were group housed (2-3/cage) and maintained in a temperature-controlled vivarium under a regular 12-hour light/dark cycle (lights on 6:00 a.m. – 6:00 p.m.). Food and water were available ad libitum except when rats were in ICSS test chambers. Rats were acclimated to their housing conditions for at least one week prior to handling and surgery. Testing occurred between 8:00 a.m. to 5:00 p.m. Monday through Friday. 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), approved all of the procedures and facilities used in the study.

2.2 Drugs

Morphine sulfate (Sigma-Aldrich) and naloxone hydrochloride (Sigma-Aldrich) salts were dissolved in 0.9% physiological saline to create solutions of 10mg/mL morphine and 1mg/mL naloxone. These were both injected subcutaneously (SC) in a volume of 0.1 ml/100 g of body weight. Drug doses were expressed as the salt. Control SC injections consisted of 0.9% physiological saline. Prazosin, and UK-14,304 were injected intra-cerebrally (IC). Prazosin

hydrochloride was dissolved in 30% dimethylsulfoxide (DMSO) and 70% distilled water. UK-14,304 was dissolved in artificial cerebrospinal fluid (aCSF). Doses of prazosin, UK-14,304 were calculated as total dose infused bi-laterally, with half of the dose infused into each hemisphere in a volume of 0.5 μ L per side. Control IC injections were either 30% DMSO in distilled water (prazosin) or artificial CSF (UK-14,304).

2.3 Surgical Cannulae and Electrode Implantation

Rats were anesthetized with isoflurane in oxygen (5% induction, 1.5-2% maintenance) and secured in a Kopf stereotaxic frame. The incisor bar was set at 3.3 mm below the interaural line for perpendicular implantations relative to the surface of the skull. Surgical instruments were cleaned with alcohol prep pads and heat sterilized in glass beads before each procedure. Stainless steel guide cannulae (SmallParts, 23 gauge) were cut at a length of 12.5 mm and rinsed with 70% alcohol. Cannula length stylets (SmallParts, 30 gauge) were prepared and rinsed in 70% alcohol along with electrodes. Unilateral, bipolar electrodes (Plastics One) were cut for an electrode length of 16 mm with the tips of the electrode probes separated by 1mm.

Six anchor screws were inserted in the skull to provide adherence anchor points for the dental cement used to hold the electrode and cannulae in place. The bilateral stainless steel guide cannulae (Small Parts, 23 gauge, 12.5 mm

length) were inserted through burr holes in the skull, with the tip of the cannulae positioned at sites 2.5 mm above the central amygdala (CeA). To position the cannulae, coordinates were measured from the flat skull surface at bregma. The cannulae coordinates were: AP -2.2. mm, ML $\pm$ 4.2 mm, DV -5.9 mm according to the atlas of Paxinos and Watson (2006). Separate groups of rats were used for diffusion control, and their guide cannulae was set at 3.5 mm above the CeA, for a final infusion target at 1 mm above the CeA target. Once the guide cannulae were positioned correctly, dental cement was applied to the posterior and lateral regions of the skull to secure the cannulae and provide space along the midline for electrode implantation. Once the dental cement was hardened, cannula-length stylets were inserted to maintain patency.

The proximity between the electrode and the CeA guide cannulae required that the electrode be inserted after securing the guide cannulae. Electrodes were implanted in the medial forebrain bundle, alternating between the left and right side of the brain for a given rat. The coordinates for electrodes were set at: AP -2.8 mm, ML $\pm$ 1.7 mm from Bregma, and DV: -7.9 mm was measured from the dura layer. Once the electrode was positioned in the brain, a small layer of dental cement was used to secure the base of the electrode where it entered the skull in place. Once set, the remaining length of the electrode outside the skull was bent at two right angles to accommodate the placement of the screw mount for the electrode away from the CeA guide cannulae. Further cement was then added to fix the electrode in this final new position. At the end of the surgical procedure,

0.2 mL of 1% Bupivacaine was topically applied around the base of the cap, followed by antibiotic ointment. Rats were housed in the vivarium and allowed to recover for one week to allow recovery before operant training.

2.4 Injection Procedure

Handling and “mock” injection procedures (where the rats were handled as during injection, but without the injector protruding past the guide cannula tip) ensured a stable baseline prior to the actual injection procedure. For mock and actual injections, animals were gently held by the investigator for 3-5 minutes during the procedure. For actual injections, dummy stylets were removed and replaced by bilateral 15 mm. injector cannulae (SmallParts, 30 gauge), which extended 2.5 mm beyond the end of the guide cannulae. The injector cannulae were connected to a 10 μ L microsyringe (Hamilton) via ~50 cm of with polyethylene tubing to allow for head motion. The microsyringe was driven by a Harvard Apparatus microsyringe pump that delivered 0.5 μ L of the drug solution through bilateral injectors over a 60 second infusion period. After infusions, the injectors were left in place for at least 90 seconds to maximize diffusion with minimal efflux up the cannula tract. Dummy stylets were immediately re-inserted into the guide cannulae following injector removal.

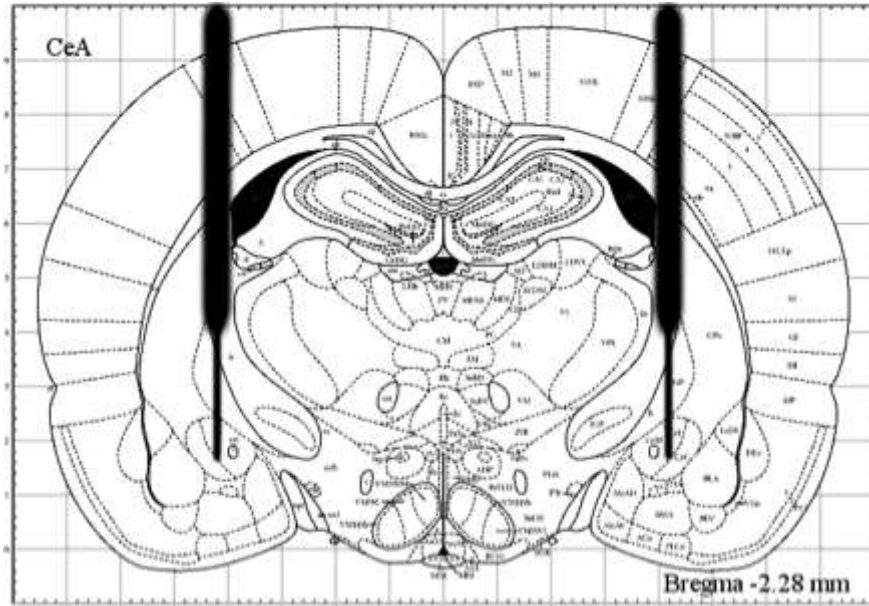


Figure 1. This schematic is a representation of the guide cannule (broad bars) and injector cannulae (thin bars) reaching their target for the CeA region. This image represents a coronal brain section -2.28 mm relative to bregma. The images are adapted from the atlas of Paxinos and Watson (2006).

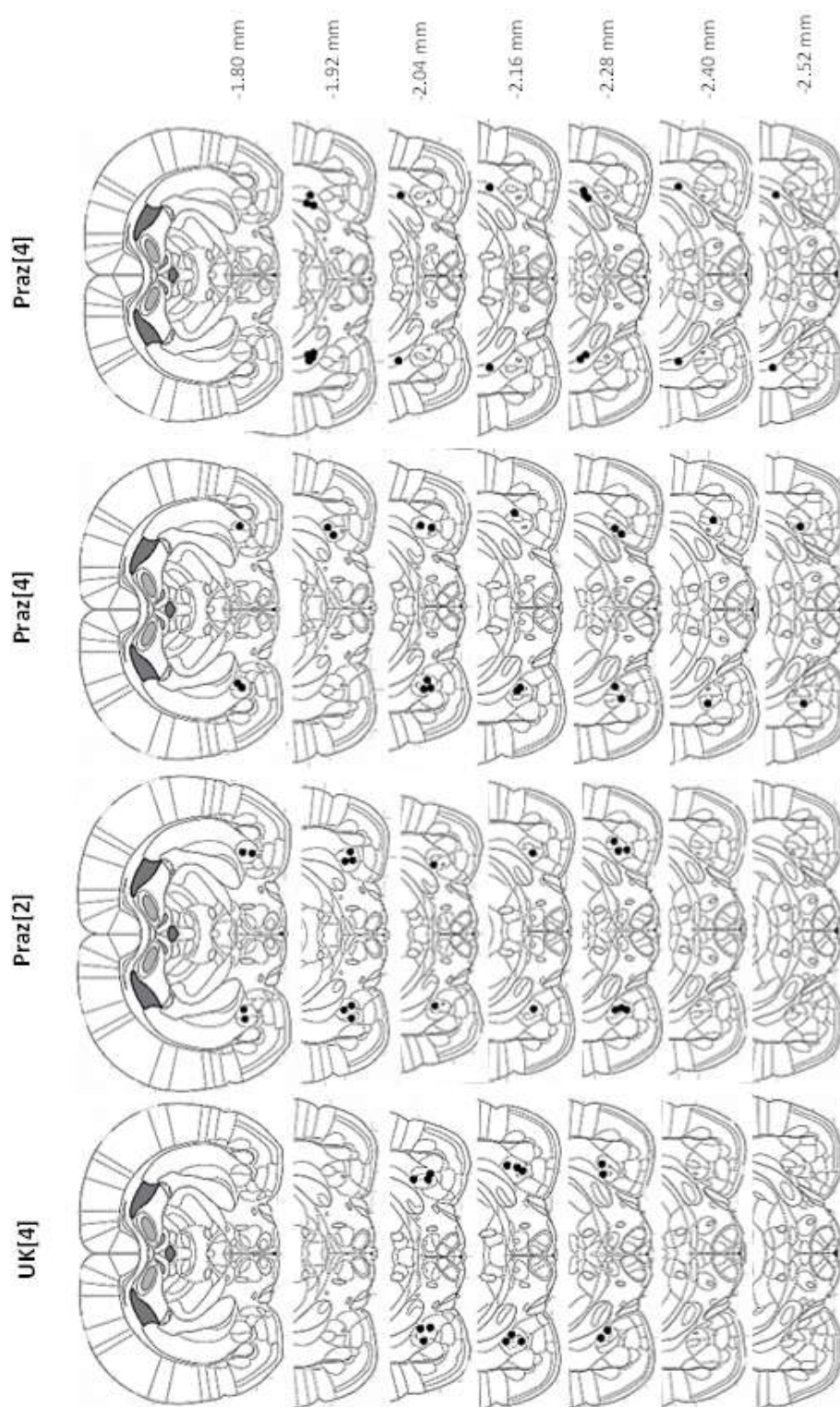
2.5 Verification of surgical cannula placements

Following the completion of testing, injector-length 15 mm stylets were secured in the cannula for at least 5 days to establish a readily visualized tract to the exact location of the injector tip. After 5 days with the stylets in place, rats were overdosed with the Euthasol (pentobarbital). Rats were tested for reflexes, and once fully unconscious but while the heart remained beating, they were transcardially perfused with 10% buffered formalin. Brains were collected and stored in 10% formalin fixative for at least 24 hours. The solution was next changed to 30% sucrose in PBS until the brain sank to the bottom of the

collection vial. Once the brains have equilibrated with the sucrose solution they were taken out, dried, and frozen in Optimum Cutting Temperature (OCT) solution. Frozen samples were coronally sectioned into 50 μm slices using a Cryostat. Slices were collected on slides and stained with cresyl violet. Sections were examined under a light microscope. The area of the terminal injector tip was found by referencing *The Rat Brain in Stereotaxic Coordinates* by Paxinos and Watson (2006). The terminal location was mapped onto the appropriate section and brain region. **Figure 2** shows the termination points of injectors with correct guide cannulae placements. **Figure 2** is representative of the full range of cannula placements although only certain groups are plotted to enhance clarity. The diffusion control histology map shows the placement distinctions between the two conditions. Only animals with correct injector placement were included in the study. Data from subjects with placement outside of the CeA were discarded.

Figure 2. Injector cannula tip termination locations for key tested drug conditions are marked by a black dot. All rats represented underwent naloxone precipitated morphine withdrawal. The drugs infused into the CeA is are shown under each tissue section set. Coronal sections are shown going from the anterior to posterior direction with coordinates from Bregma listed on the right. Non-Control rats with injector tips outside of the CeA were excluded from the study. The diffusion control rats had cannula injector tips located ~ 1 mm. above the CeA target. Images adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 2006).

Diffusion Control



2.6 Discrete – Trial brain stimulation reward procedure

The discrete-trial current-threshold intracranial self-stimulation (ICSS) paradigm using the psychophysical method of limits (Kornetsky and Esposito 1979) was used to elucidate the effects of drugs on reward function. This technique can characterize the drug-induced states of euphoria-like or dysphroia-like signs by alternations in brain stimulation reward (BSR) thresholds under drug conditions compared to a stable defined baseline. The electrode implantation during surgery is key for ICSS. Electrodes are aimed at the medial forebrain bundle (MFB), and an animal's willingness to self-stimulate is taken as a sign of correct electrode placement. Although alternate BSR techniques measure response rates, this study used a discrete-trial ICSS procedure that required few total responses. This allowed reliable measurements of BSR with minimal confound by non-specific motoric effects of drugs (Markou and Koob, 1992).

There were several training stages to transition rats to discrete trial ICSS. The parameters of ICSS utilized 100 Hz sinusoidal waves with pulse train duration of 250 ms. while varying current intensity amplitude. All rats were trained while drug naïve. After a recovering from surgery rats are introduced to an operant chamber with a movable wheel attached to one wall. Upon their first exposure to the apparatus they receive a response-contingent stimulus of 120 μ A upon every $\frac{1}{4}$ turn of the wheel. Independently self-administering 100 successful rewards within a three minute window at a given current was the criteria for moving rats to the next stage of training. If a rat did not meet this criteria at the

initial stimulation amplitude, intensity varied by ± 10 -30 μA to seek the rat's preferred current.

Once passing this first stage, the animals moved to a 2nd more complex stage in which they were given a non-contingent stimulus pulse by the computer, and then allowed 7.5 sec to respond for another contingent stimulus of the same intensity. In successive steps of training, the rats were conditioned to wait with increasing delays of 1, 5, 10, and 15 seconds after a successful response before receiving the next non-contingent pulse and response opportunity. The criterion for progression across stages of increasing delay was at least 40% correct responses (within 7.5 sec) out of 100 trials. Responses during the 1-15 second "timeout" interval did not result in a reward, but instead delayed the onset of the next non-contingent stimulus to condition the animals to minimize response rates prior to moving to the final stage of ICSS training.

In the 3rd stage of training, BSR thresholds were measured by varying stimulus intensity using the psychophysical method of limits (Cornsweet 1962). As in the 2nd stage of training, the trial started with a non-contingent pulse, with initial intensity set at 30 μA higher than their mean reward threshold during the preceding threshold determination session. This is done to get an accurate measure of lower threshold by starting the descending series at an elevated point. As in the 2nd stage of training, the rat had 7.5 seconds to make a $\frac{1}{4}$ turn of the wheel to receive a second pulse of electrical stimulation that was equal in

intensity to the first. If the rat responded by spinning the wheel in this time frame a positive response was recorded, and a negative response was recorded if not.

The end of each trial was interspersed with inter-trial intervals ranging from 7.5-12.5 seconds and averaged 10 seconds. A response during the inter-trial interval resulted in a ~10 second delay before the start of the next trial, thereby discouraging extra responding and ensuring that positive responses were truly contingent upon having felt the stimulation at the given intensity, and desiring another stimulus of the same intensity. Stimulus intensities varied in amplitude in two sets of alternating descending and ascending trials with a step size of 5 μ A. Each trial block at a given stimulus intensity consisted of three trials. The threshold for each ascending/descending series was taken as the midpoint between intensities where a rat responded at least 2/3 times and the intensity at which it responded fewer than 2 times. Before the series would switch from descending to ascending, at least 2 consecutive stimulus intensities with fewer than 2 responses were required (to switch from ascending to descending, two consecutive intensities with 2 or more responses was required). **Figure 3** gives a hypothetical example of how such thresholds are calculated. A final mean threshold is calculated as an average of the four thresholds determined for each ascending/descending series.

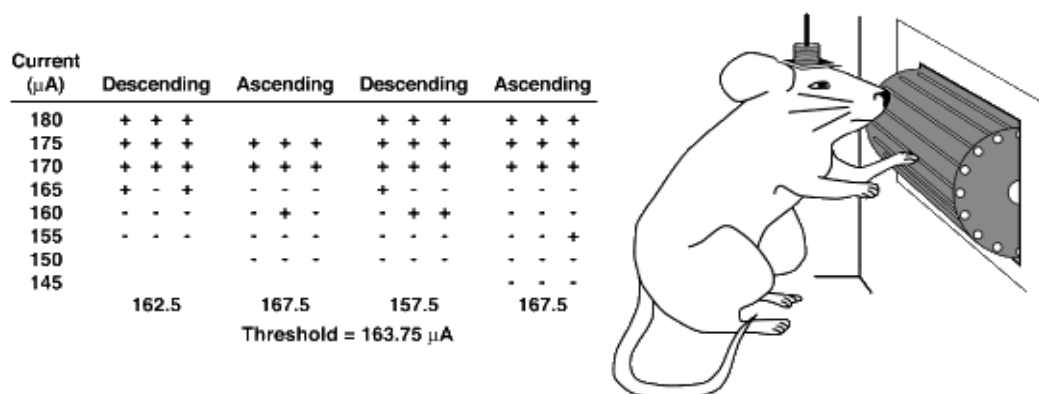


Figure 3. Example of responses to a discrete trial ICSS procedure. The table represents a hypothetical model of how thresholds are established with alternating trial currents over a given session. Image adapted from *Neurobiology of Addiction* (Koob and Le Moal, 2006).

2.7 Experimental Design

Rats were trained for at least three weeks to reach a point where their mean thresholds varied by less than 15% for a given week of training. Rats were trained twice daily with “AM” (mid-morning) and “PM” (early afternoon) runs separated by ~4 hours in the ICSS procedure. Once stability was noted for a group of at least 4-8 rats, a baseline was established.

Baseline Phase: Over a 3-4 day baseline period, rats received a SC injection of saline after their AM training session. Approximately 3 hours and 45 minutes after this injection, rats were given mock infusions in which they were gently restrained as during an actual injection, their stylets removed, and they were held in place and exposed to the noise of the motor of the microsyringe pump for 2-3 minutes. Stylets were replaced after the mock infusions. Rats were

given a second SC injection four hours after the first, immediately prior to their PM training session.

Testing Phase: The testing procedure is based on previous work that determined the optimal conditions and delay for precipitated withdrawal from acute morphine (Schulteis et al., 1997; Esterling and Holtzman, 1997; Liu and Schulteis, 2004; Chang and Schulteis 2011). The same schedule for baselining was used during acute testing days. Following their regular AM run, testing rats were given a SC injection of either 0.9% saline or morphine at 10 mg/kg. Three hours and 45 minutes after the initial injection, rats were given bilateral infusions of 0.5 μ L of either vehicle solution or drug (UK 14304 or Prazosin). Infusions lasted approximately 3-5 minutes with rats gently restrained. Following the infusion, rats were placed back in their cages for 10 minutes. At four hours after the morning SC injection rats were given a second SC injection of either 0.9% saline or naloxone (1 mg/kg). Rats were given their PM ICSS test run immediately following this final SC injection. Rats who did not finish all four columns of the ICSS run were excluded from the study.

Table 1. Abbreviations used to indicate drug conditions in various experimental groups. The experimental design is outlined via the time frames shown for injections. Time is measured from the point where the initial morphine or vehicle injection is given. Intracranial infusions of are given at T = 3 hr and 45 min. Injections of naloxone or vehicle are given at 4 hours post initial morning injection. The abbreviations below will indicate the drug given directly correlated to the time line shown. For example Mor-Praz[2]-Nal indicates that the subject received an initial morning injection of morphine at 10 mg/kg, then an intracranial infusion of 2ug of prazosin at 3 hr and 45 min, and lastly an afternoon injection of naloxone at 1 mg/kg. Morphine and naloxone concentrations were not varied in this experiment.

Test Day	T= 0	T= 3 hr 45 min	T= 4 hr
MOR-NAÏVE	Vehicle SC	Test Drug/Vehicle IC	Naloxone/Vehicle SC
ACUTE	Morphine SC	Test Drug/Vehicle IC	Naloxone/Vehicle SC
Abbreviations	Veh or Mor	Veh, UK[4], Praz[2], or Praz[4]	Veh or Nal

Table 1 shows the drug conditions given within the timeframe of the experiment.

Table 2 shows the raw average baseline data for all tested rats. There were no variations in handling of rats in different groups during the baseline procedure. To the greatest extent possible, rats were counterbalanced to minimize any group differences in baseline threshold. The last diffusion control group was done as part of the last set of tests, as it was only a necessary group to run after establishing a dose-dependent effect of prazosin; consequently, counterbalancing of baseline threshold was not possible in this group.

Table 2. Absolute baseline BSR thresholds and response latencies are listed for all drug groups tested. The rats were all treated with the same conditions during the baseline procedure. Rats were given morning SC injections of 0.4 mL isotonic saline. 3 hours and 45 minutes later, they were given mock infusions. During mock infusions, rats were handled in the presence of the infusion pump motor noise while the stylet wire within their cannula were manipulated. After 3-5 minutes of mock infusions, rats were placed back in their home cage. After another 10 minutes brain reward thresholds were tested. All values above are averages. DC refers to Diffusion Control groups, where cannula were placed 1 mm above the normal placement for CeA infusions.

Drug Conditions	AM Baseline Thresholds (μ A)	PM Baseline Threshold (μ A)	PM Baseline Latency (Seconds)
Veh-Veh-Veh (n=10)	74.22 $\pm$ 9.91	79.79 $\pm$ 11.16	2.48 $\pm$ 0.11
Veh-Veh-Nal (n=18)	74.87 $\pm$ 8.92	79.89 $\pm$ 9.49	2.85 $\pm$ 0.10
Veh-UK[4]-Nal (n=10)	68.96 $\pm$ 6.86	73.44 $\pm$ 8.14	2.89 $\pm$ 0.22
Veh-Praz[4]-Nal (n=11)	76.77 $\pm$ 12.96	81.78 $\pm$ 13.32	2.61 $\pm$ 0.15
Mor-Veh-Veh (n=10)	69.34 $\pm$ 5.52	74.77 $\pm$ 6.58	2.58 $\pm$ 0.10
Mor-Veh-Nal (n=17)	68.13 $\pm$ 8.67	73.09 $\pm$ 9.37	2.75 $\pm$ 0.15
Mor-UK[4]-Nal (n=10)	77.71 $\pm$ 9.84	85.43 $\pm$ 10.95	2.54 $\pm$ 0.15
Mor-Praz[2]-Nal (n=10)	67.02 $\pm$ 5.80	71.34 $\pm$ 6.47	2.65 $\pm$ 0.11
Mor-Praz[4]-Nal (n=11)	70.23 $\pm$ 6.80	77.57 $\pm$ 8.65	2.49 $\pm$ 0.10
Mor-Praz[4]DC-Nal (n=10)	58.78 $\pm$ 4.57	62.94 $\pm$ 5.62	2.50 $\pm$ 0.14

2.8 Statistical Analysis

Mean thresholds for the testing trials were expressed as a percent of the threshold obtained during the baseline (mock infusion) sessions. Results were analyzed through one-way or two-way analysis of variance (ANOVAs), as appropriate. A constant level of $p < 0.05$ was used to gauge for statistical significance. Bonferroni corrections were applied when multiple comparisons of two means followed overall significant outcomes in the ANOVA. JMP 10.0 for Macintosh (SAS, Cary NC) was used to analyze data.

3 Results

3.1 Comparing morphine naïve Veh-Veh-Nal control groups

Veh-Veh-Nal control groups receiving intracranial infusions of either aCSF or 30% DMSO in distilled water prior to naloxone were compared to each other in a one factor ANOVA and were found not to differ ($F[1,16] = 0.44$, $p > 0.50$), and therefore were combined into a single Veh-Veh-Nal control group for further comparisons.

3.2 Comparing acute morphine withdrawal (Mor-Veh-Nal) control groups

When naloxone was administered 4 hr post-morphine, there was a significant increase in threshold in the Mor-Veh-Nal condition regardless of whether the IC infusion was aCSF or 30% DMSO, and these groups did not differ from each other ($F[1, 15] = 1.59$, $p > 0.20$). Therefore these groups were also combined into a single Mor-Veh-Nal group for further analyses.

3.3 Naloxone following morphine increases BSR thresholds without changing response latencies

As shown in **Figures 4 and 5**, the combination of morphine and naloxone significantly elevated thresholds, as revealed by a 2 x 2 ANOVA with morphine condition (Veh, Mor) and naloxone condition (Veh, Nal) as factors. There was a

significant main effect of morphine condition ($F[1, 51] = 9.97, p < 0.005$), naloxone condition ($F[1, 51] = 14.84, p < 0.0005$), and morphine x naloxone interaction ($F[1, 51] = 14.68, p < 0.0005$). Post-hoc comparisons of Mor-Veh-Nal groups to all other control conditions (Mor-Veh-Veh, Veh-Veh-Nal, Veh-Veh-Veh) were significant (all p 's < 0.5 , Bonferelli Corrected). A similar 2 x 2 ANOVA for response latency revealed no significant main effect or interaction (all F 's < 2.03 , p 's > 0.16).

3.4 Prazosin and UK 14,304 do not alter BSR thresholds or response latencies on their own

The highest tested doses of UK 14,304 or prazosin (4 μ g) did not alter thresholds ($F[3, 45] = 0.46, p > 0.70$) or response latencies ($F[3, 45] = 1.8815, p > 0.14$) when administered prior to naloxone but in the absence of morphine pretreatment (Veh-Praz[4]-Nal, Veh-UK[4]-Nal groups compared to Veh-Veh-Nal, Veh-Veh-Veh controls).

3.5 Dose dependent attenuation of naloxone-precipitated BSR threshold increases by prazosin

As show in **Figure 4**, when prazosin (2 or 4 μ g) was infused prior to naloxone in morphine-pre-treated rats, it dose-dependently attenuated the naloxone-precipitated threshold elevation ($F[2, 35] = 4.58, p < 0.025$). Post-hoc

comparisons (Bonferroni- corrected) revealed that this effect was significant only at the 4 μ g dose ($p < 0.05$ vs. Mor-Veh-Nal control). This effect was a partial but incomplete reversal, as the Mor-Praz[4]-Nal group still differed significantly from the Mor-Veh-Veh control group ($p < 0.05$). There was no difference in latencies between morphine groups tested with prazosin (Mor-Praz[2]-Nal, Mor-Praz[4]-Nal) and the control withdrawal group (Mor-Veh-Nal) ($F[2, 35] = 0.33$, $p > 0.70$).

The effect of prazosin on BSR thresholds was specific to intra-CeA infusion of Praz[4], since a diffusion control group where this dose was injected 1 mm above the CeA was different from the Mor-Praz[4](CeA)-Nal group, but not from the Mor-Veh-Nal group (see **Figure 4**).

3.6 UK 14,304 did not significantly alter naloxone-precipitated BSR threshold increases.

In contrast, as shown in **Figure 5**, UK 14,304 administered prior to naloxone in morphine pre-treated rats did not significantly reverse the naloxone precipitated threshold increase ($F[1, 23] = 2.81$, $p > 0.10$). There was no significant change in latencies as well ($F[1, 23] = 0.21$, $p > 0.5$).

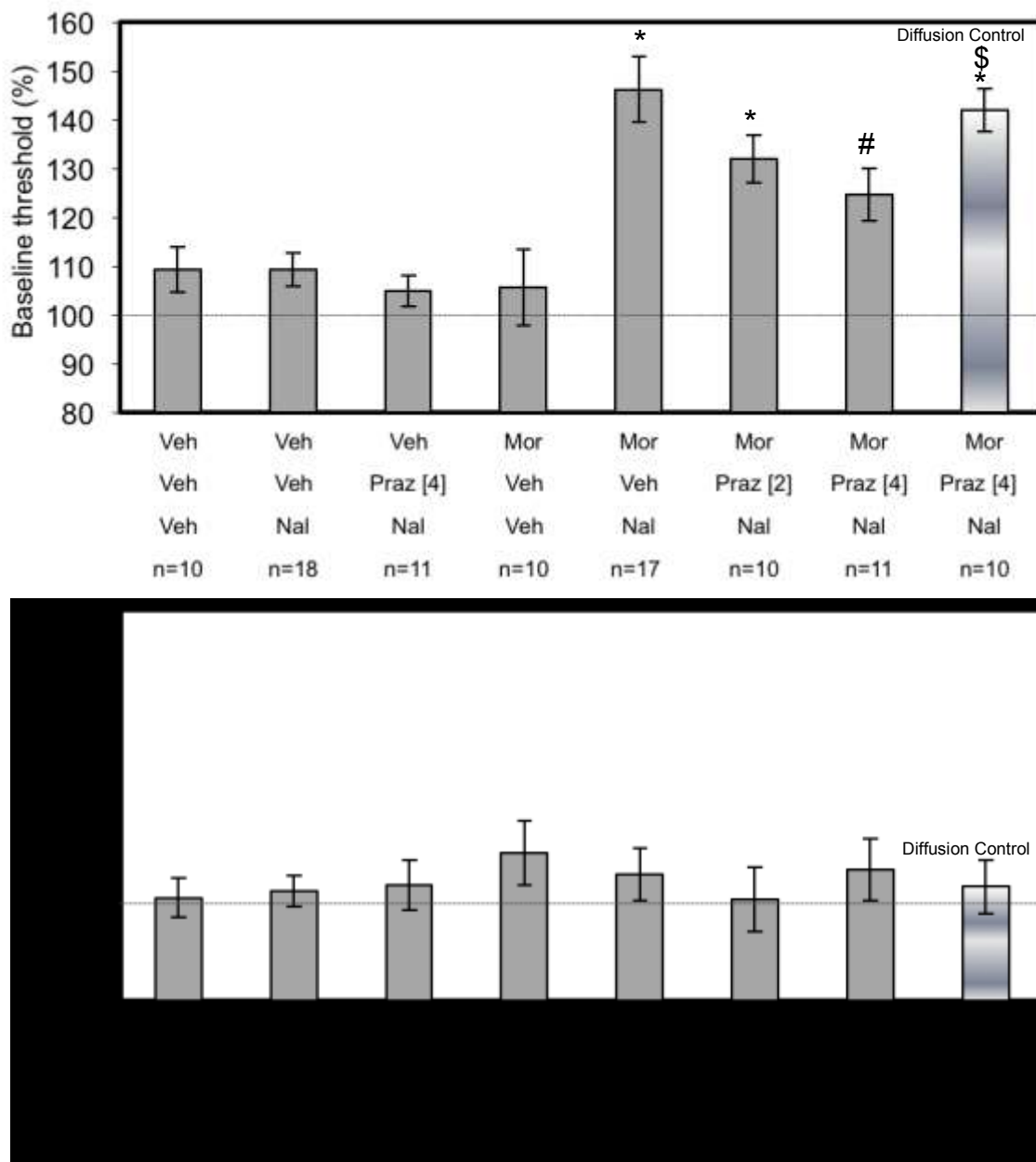


Figure 4. Effects of intra CeA infusion of α_1 adrenergic antagonist prazosin on brain stimulation reward threshold and response latencies. All rats received a SC injection of either morphine (10 mg/kg) or isotonic saline vehicle. After 3 hr 45 minutes, rats received an IC administration of 2 or 4 μ g of Prazosin or vehicle (half of the dose in each hemisphere in 0.5 μ L solution). After another 15 min, SC naloxone (1.0 mg/kg) or saline vehicle was administered before testing the brain reward threshold. The last column represents prazosin infusion into the diffusion control site, 1 mm above the site of interest. Data represents mean ($\pm$ S.E.M) percent of baseline threshold.* shows significant difference from Mor-Veh-Veh groups. # shows significant difference from Mor-Veh-Nal group. \$ shows significant difference from Mor-Praz [4]-Nal group that directly targeted prazosin in the CeA. Significance levels held constant at $P < 0.05$.

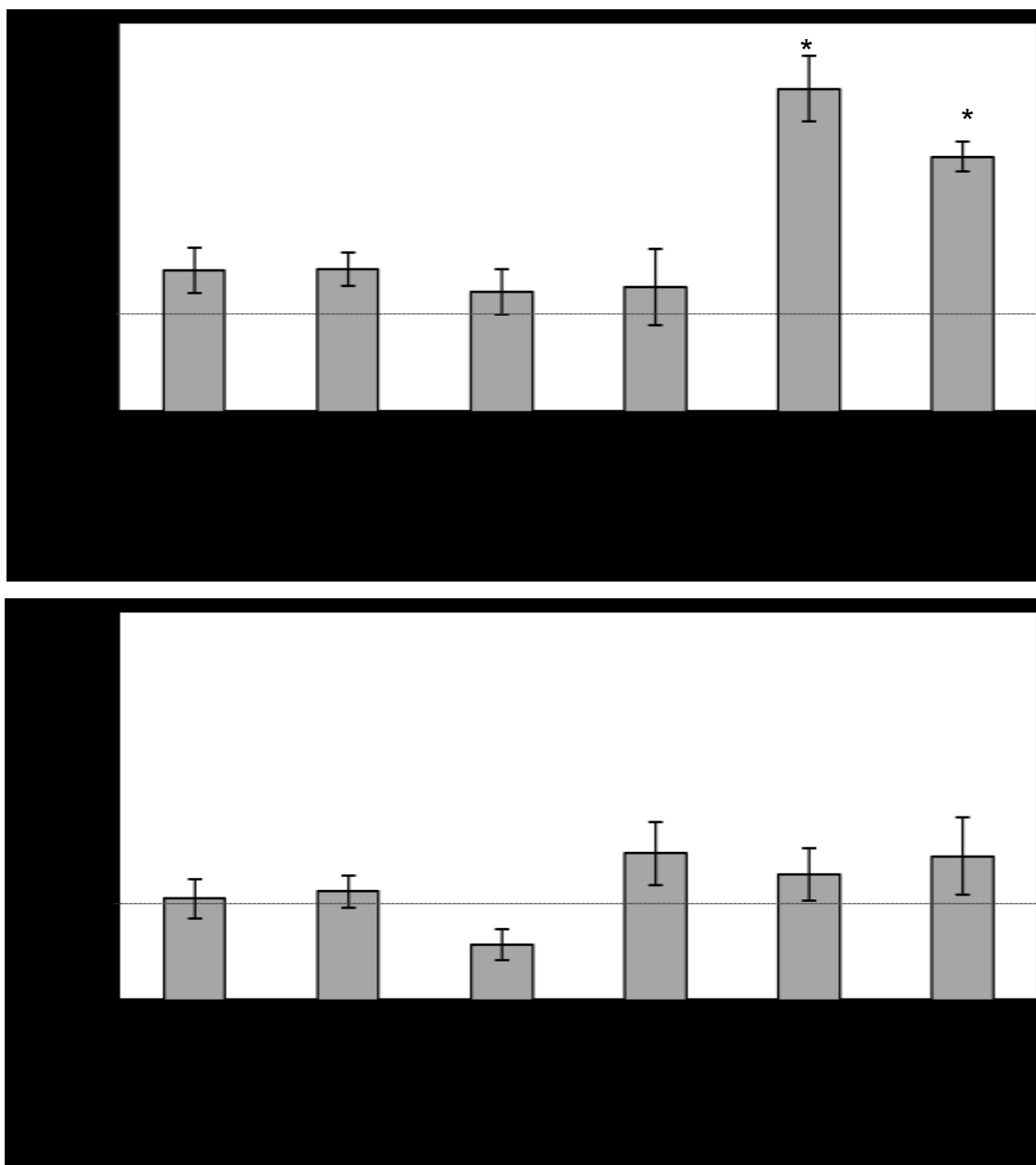


Figure 5. Effects of intra CeA infusion of α_2 adrenergic agonist UK 14,304 on brain stimulation reward threshold and response latencies. All rats received a SC injection of either morphine (10 mg/kg) or isotonic saline vehicle. After 3 hr 45 minutes, rats received an IC administration of 4 μ g of UK 14,304 or vehicle in 1 μ L solution. After another 15 min, SC naloxone (1.0 mg/kg) or saline vehicle was administered before testing the brain reward threshold. Data represents mean ($\pm$ S.E.M) percent of baseline threshold.* shows significant difference from Mor-Veh-Veh groups. Significance levels held constant at $P < 0.05$.

4 Discussion

The results show that the α 1-adrenergic antagonist prazosin infused into the CeA dose dependently attenuated brain reward deficits during acute naloxone-precipitated morphine withdrawal. A total dose of 4 μ g of prazosin infused bilaterally into the CeA significantly reduced average BSR thresholds from 146.17% to 124.55% when compared to the morphine withdrawal control group (Mor-Veh-Nal). There were no significant differences seen in response latencies between prazosin and control groups, indicating that these results are independent of motor influences of the drug. The diffusion control tests failed to show a reduction, indicating that the action of prazosin was specific to the CeA. The α 1 adrenergic receptors in the CeA seem to have a partial role in mediating the dysphoric effects of opioids withdrawal. These partial attenuation results indicate a potential secondary role of NE in the CeA, as prior studies by Chang found that CRF antagonists produce a complete reversal of naloxone precipitated morphine withdrawal when injected into the CeA.

The α 2 agonist UK 14,304 also slightly reduced acute withdrawal signs measured in the ICSS paradigm, but insignificantly, from 146.17% threshold to 132.31%, again without affecting response latencies. A single dose of 4 μ g was used to test for an ICSS related effect on morphine withdrawal. No higher doses were tested because 4 μ g is already a very high dose when compared to the significant effects on anxiety-like behavior observed by Powell and colleagues (2011) with systemic administration of 20 μ g/kg (roughly 7 μ g for a 350 gm rat). In contrast a 100 μ g systemic dose of prazosin was needed to see attenuation of

acute naloxone-precipitated morphine withdrawal in the ICSS paradigm, while the present study found significant results at just 4 μ g intra-CeA (Archer et al., 2013). These results suggest that the effects of systemic α 1 antagonists may be mediated, at least in part, in the CeA, but that disruption of NE release through systemic α 2 agonist treatment may exert effects on opioid withdrawal through regions other than the CeA. Within the amygdala, there are both α 1 and α 2 receptors, but less expression of α 2 subtypes (McCune et al., 1993), suggesting a plausible explanation for our findings of greater potency of prazosin than UK 14,304 after intra-CeA infusion.

Studies using NE and CRF reducing drugs administered systemically have shown that both stress transmitters contribute to the expression of affective components of opioid withdrawal symptoms. Powell et al. (2011) found that NE reducing drugs fully reverse anxiety-like signs of acute morphine withdrawal in the elevated plus maze paradigm, while CRF reducing drugs only provided a partial attenuation. Conversely with ICSS thresholds, CRF reducing agents were able to yield a complete reversal in acute morphine withdrawal while α 2 agonist NE reducing agents attenuated, but did not fully reverse, dysphoric withdrawal signs following repeated morphine administration (4 days of daily morphine), but were less effective in attenuating withdrawal after acute morphine intoxication (Powell et al., 2011). Therefore it seems that NE may play an increasing role in the dysphoric-like symptoms of precipitated withdrawal after repeated, chronic, opioid experience.

For the CeA specifically, Chang found that the CRF antagonist antalarmin infused into the CeA 10 min (Chang 2011). prior to acute morphine withdrawal dose-dependently and fully reversed dysphoria-like signs of opioid withdrawal measured through ICSS. In the present study, intra-CeA prazosin only partially attenuated withdrawal measured in the same paradigm. Combined with the results of systemic administration of CRF and NE antagonists, our results suggest that CRF may be critical to the initial expression of dysphoria-like signs of withdrawal from acute morphine dependence, whereas NE becomes increasingly recruited after repeated administration. CRF and NE have an interconnected relationship that may explain the overlap of role in producing affective symptoms of withdrawal.

Both CRF and NE work to mediate stress responses during drug withdrawal. There are several regions of the brain that display interconnection between the two NT systems. The locus coeruleus synapses onto the forebrain through NE, which triggers CRF neurons to release CRF onto the brainstem. NE in the brainstem in turn projects forward to regions of the “extended amygdala” including the CeA, and evidence suggests that these projections are critical to the aversive emotional consequences of opioid withdrawal (e.g. Aston-Jones et al., 1999). This has been hypothesized as a NE-CRF-NE feed-forward system (Koob, 1999; Kravets et al., 2013). This feed-forward system may work as a mechanism for which organisms rapidly mobilize the hypothalamic pituitary adrenal axis as well as brain stress circuitry in response to environmental threats.

The pathological over-activation of this system has been hypothesized to underlie anxiety disorders from social phobia to post-traumatic stress disorder (Koob 1999; Jacobsen et al., 2001). Lesion studies which eliminated the locus coeruleus as a source of NE found that it was not critical for the aversive affective symptoms of opioid withdrawal (Caille et al., 1999).

Our results in combination with Chang would suggest that, for dysphoria-like signs of opioid withdrawal, this feed-forward system may be initially recruited in acute opioid dependence through adaptation in CRF release in the CeA, which in turn may subsequently drive brainstem NE to feed-forward back into the extended amygdala upon repeated opioid experience. This is evident by the full reversal of negative affective state of opioid withdrawal measured via ICSS when infusing CRF-1 antagonists into the CeA, compared to the partial reversal derived from infusion of $\alpha 1$ adrenergic antagonists. Thus, although CRF systems appear to be predominant in the initial dysphoria-like response to withdrawal from acute opioid dependence, NE systems may still represent a viable target for therapeutic intervention in chronic users.

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