UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the Dynorphin/Kappa-opioid Receptor System and CREB in the Compulsive Behaviors Associated with Extended Access to Methamphetamine

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

Master of Science

in

Biology

by

Jessie Margolis

Committee in charge:

Professor George F. Koob, Chair
Professor Eduardo Macagno, Co-Chair
Professor Brenda Bloodgood

2014

This Thesis of Jessie Margolis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California, San Diego
2014

DEDICATION

This thesis is dedicated to Judith, Morton, Nina and Todd Margolis, for their love and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CREB	Cyclic Adenosine Monophosphate Response Element-binding Protein
FR	Fixed-ratio
GABA	Gamma-aminobutyric acid
IHC	Immunohistochemistry
IR	Immunoreactivity
κOR	Kappa-Opioid Receptor
LgA	Long-access
MID	Mean Integrated Density
NAc	Nucleus Accumbens
Nor-BNI	nor-Binaltorphamine
p-CREB	Phospho-CREB
PR	Progressive-ratio
ShA	Short-access
VTA	Ventral Tegmental Area

ACKNOWLEDGEMENTS

In addition to Dr. Koob and the members of my committee, I would like to acknowledge the following members of Koob Lab who made completion of this thesis possible: Yanabel Grant, Elena Crawford, Dr. Joel Schlosburg, Dr. Olivier George, Eva Zamora-Martinez, and Don Vuong. Special acknowledgement is made to Dr. Timothy Whitfield Jr., who acted as my research mentor and assisted me with experimental design and data collection.

Experiment 2, in part, is currently being prepared for submission for publication of the material. Whitfield Timothy W. Jr., Grant Yanabel, Margolis Jessie, Koob George F. The thesis author was a researcher and co-author of this material.

ABSTRACT OF THE THESIS

The Role of the Dynorphin/Kappa-opioid Receptor System and CREB in the Compulsive Behaviors Associated with Extended Access to Methamphetamine

by

Jessie Margolis

Master of Science in Biology

University of California, San Diego, 2014

Professor George F. Koob, Chair
Professor Eduardo Macagno, Co-Chair

Psychostimulant addiction is a chronically relapsing disorder characterized by escalated drug intake and persistent drug craving, which are compulsive behavioral effects due to negative affective states experienced during withdrawal. When endogenous opioid dynorphin binds to kappa-opioid receptors (κORs) in the mesolimbic system, transmission of dopamine to the nucleus accumbens (NAc) is decreased. It is theorized

that increased activity of the dynorphin/κOR system mediates the dysphoric component of psychostimulant withdrawal and thus facilitates the transition from occasional psychostimulant use to chronic dependence. This thesis examines two aspects of how upregulation of the dynorphin/κOR system drives the transition toward methamphetamine dependence. Dynorphin activates κORs, which leads to increased activity of transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) within the NAc; in turn, increased CREB activity leads to expression of more dynorphin. Experiment 1 explored the relationship between elevated NAc CREB activity and the compulsive drug-–taking behavior associated with extended access to methamphetamine. Immunoreactivity of phospho-CREB within the NAc core and shell was compared to access condition (1 h limited- or 6 h extended-access) and overall escalation of methamphetamine self-administration. The positive correlation between NAc phospho-CREB and methamphetamine intake implicates mesolimbic CREB activity in compulsive methamphetamine-taking behavior. Experiment 2, which examined the effects of κOR antagonist nor-Binaltorphamine (nor-BNI) on re-escalation of drug-taking and reinstatement of drug-seeking, showed that the compulsive behaviors associated with chronic methamphetamine abuse are reversible. The combined results of this thesis preliminarily identify NAc CREB as a potential therapeutic target for treatment of pre-existing methamphetamine addiction.

Introduction

Substance abuse is a high profile issue worldwide due to both its direct health effects on the individuals that take drugs and also the magnitude by which it affects society as a whole. Death by overdose, increased susceptibility to and transmission of infection, and health problems including cardiovascular disease, respiratory failure and comorbid psychiatric disorders are only a few of the direct consequences of drug abuse and addiction. (Harwood et al. 1998) Furthermore, the demand for illicit substances fuels illegal trafficking and drug-related violence, addicted individuals are often inconsistent members of the workforce, and dependent users are prone to accidentally harming themselves and others while under the influence (Harwood et al. 1998). Taking all of these effects on society into account, it was estimated that substance abuse and dependence in the United States alone cost society over 100 billion dollars in 1998 (Harwood *et al.* 1998). Methamphetamine is a highly addictive psychostimulant drug that has a particularly negative impact on both health and society as a whole. Methamphetamine abuse is an international health problem, particularly in North America and Asia (Kosten and Newton, 2012). It is estimated that 33 million people worldwide abuse the drug, and there are over 600,000 new abusers documented each year in the United States alone (Kosten and Newton, 2012). Methamphetamine use has a significant impact on cardiovascular health and has been classified in coroners' reports as direct cause of death in 68% of lethal stimulant overdose cases (Kosten and Newton, 2012.) Apart from its negative effects on physical health, methamphetamine abuse puts human users at high risk for mental health disorders such as psychosis, depression and

anxiety (Kosten and Newton 2012, Koob and Le Moal 2006). Methamphetamine abuse is characterized by compulsive behaviors including significant preoccupation with obtaining the drug, repetitive seeking and taking the drug, and loss of control over drug intake. These compulsive behaviors meet the American Psychiatric Association's criteria for full-blown drug addiction (APA DSM IV-TR 2000). The compulsive aspects of methamphetamine abuse are highly treatment-resistant, and the neuropharmacology of compulsive methamphetamine self-administration is not well understood (Koob and Le Moal, 2006). As such, it is important to investigate the neurobiological causes of compulsive methamphetamine use in order to establish a starting point for exploration of therapeutic options to treat methamphetamine addiction.

Before treatment options for methamphetamine dependence can further be considered, it is imperative to understand what makes it so addictive. Methamphetamine is classified as a psychostimulant drug, meaning that it increases physical and mental stimulation. Other psychostimulants, such as cocaine and other amphetamine-derivatives, act in mechanistically similar ways to methamphetamine and exhibit similar stimulant effects; therefore, examination of psychostimulants in general provides us with a broader understanding of their addictive properties (Snyder and Coyle 1969, Heikkila 1974). The unique profile of psychostimulant withdrawal is most likely responsible for psychostimulant users' characteristic compulsive drug-taking and -seeking behavior as well as their high rate of relapse (Koob and Le Moal, 2006). The motivational symptoms of psychostimulant withdrawal include depression, anxiety, craving for the drug and general psychological malaise. Although the severity of these withdrawal symptoms wanes with prolonged abstinence, craving for the drug remains indefinitely (Koob and Le

Moal, 2006). What causes these withdrawal symptoms, and what makes their actions so persistent? Extensive investigation of how chronic psychostimulant use leads to neuroadaptations in the brain has guided addiction neurobiologists in a promising direction to answer this question.

Transmission of monoamine neurotransmitter dopamine within the mesolimbic dopamine system mediates the euphoria that occurs in response to both natural rewards and drugs of abuse (Wise and Bozarth, 1984). The mesolimbic system consists of dopaminergic neurons in the ventral tegmental area (VTA) and their projections onto structures that make up the ventral striatum region (Wise and Bozarth, 1984). Of particular interest is the projection from the VTA to the nucleus accumbens (NAc). The NAc consists of two distinct regions, the core and the shell (Voorn et al. 1989). Dopamine transmission from the VTA to the NAc regulates the excitability of inhibitory GABAergic medium spiny neurons in the NAc, and it has been repeatedly demonstrated that the acute increase of dopamine transmission in this pathway facilitates the rewarding effects of psychostimulants (di Chiara and Imperato 1988, Meredith 1999). Increased transmission of dopamine mediates drug dependency over time in response to chronic disruption of the user's physiological processes. In order to prevent the homeostatic disruption that occurs with increased and prolonged dopamine transmission to the NAc, the body downregulates dopamine release from VTA presynaptic terminals (Gawin and Ellinwood 1989, Koob and Le Moal 1997). This chronic reduction of mesolimbic dopamine transmission alters the once-pleasurable effects of the drug experienced by the user. While the occasional user's psychostimulant use is positively reinforced by increased mesolimbic dopamine transmission in immediate response to taking the drug,

the frequent user's chronically reduced mesolimbic dopamine negatively reinforces drug use, causing the psychologically uncomfortable symptoms that characterize withdrawal (Koob and Le Moal 2006). Only increased psychostimulant intake can keep these withdrawal symptoms at bay, so the user will compulsively take more whenever it is available (Koob and Le Moal 2006). Since decreased dopamine transmission drives the psychostimulant abuser's primary motivation from seeking pleasure to avoiding withdrawal, it is theorized to facilitate the user's transition to psychostimulant addiction (Koob and Le Moal 2006). Still unaccounted for in this theory is the neurobiological switch that alters dopaminergic activity within this reward circuit to precipitate withdrawal.

Further investigation of the cellular and molecular mechanisms that regulate dopamine transmission within mesolimbic system brings us closer to understanding how chronic use of methamphetamine produces withdrawal. Dopamine transmission to the NAc is modulated by endogenous opioid peptides. One of these opioid peptides, dynorphin, mediates negative reinforcement of drug use by indirectly inhibiting dopamine transmission to the NAc (Shippenberg and Rea 1997). Dynorphin selectively activates kappa-opioid receptors (κORs) located on the presynaptic axon terminals that innervate the NAc (Chavkin 1982). The majority of these innervations are located at the NAc shell region (Jones et al. 1996, Svingos et al. 1999). κOR activation by dynorphin within the NAc shell inhibits dopamine release from the VTA terminals (Di Chiara and Imperato 1988). Chronic self-administration of psychostimulants has been demonstrated to increase production of dynorphin precursor prodynorphin in the mesolimbic system in rats (Cole *et al.* 1995, Smiley *et al.* 1990). As such, it is hypothesized that chronic use of

psychostimulants increases dynorphin expression and κOR activity within the mesolimbic system, which in turn suppresses dopamine transmission to the NAc, causing the negative affect that characterizes withdrawal (Koob and Le Moal 2006).

Dysphoria, anhedonia and anxiety accompany this κOR-mediated reduction of dopamine transmission; these elements of negative psychological disposition characterize psychostimulant withdrawal (Koob and Le Moal 2006). It has been demonstrated that κOR activation by endogenous and exogenous agonists in rodents produces aversive effects measured by various behavioral assays; including conditioned place aversion, forced-swim-test-induced immobility and learned helplessness tests (Bechara and van der Kooy 1987, Newton et al. 2002, McLaughlin et al. 2003, Bruchas et al. 2007, Land et al. 2008). Both pretreatment of stress-exposed mice with κOR antagonist nor-Binaltorphimine (nor-BNI) and knockout of dynorphin precursor peptides have been shown to significantly decrease stress-response and depression behaviors; these results confirm that κOR activation is involved in psychological states similar to those that characterize psychostimulant withdrawal (Newton et al. 2002, McLaughlin et al. 2003, Bruchas et al. 2007, Land et al. 2008). Furthermore, established methods of stress induction have been demonstrated to increase endogenous production and release of dynorphin in the mesolimbic system in various rodents (Nabeshima et al. 1992, McLaughlin et al. 2003). Interpreted together, the results of these stress-related experiments indicate that not only does increased κOR activity mediate stress-response behaviors, but stress itself exacerbates further κOR activation by upregulating dynorphin production (Wee and Koob 2010, Bruchas et al. 2009).

The effect of κOR activity on compulsive psychostimulant self-administration depends on whether the user is dependent on the drug. Clinical observations in humans show that the controlled psychostimulant use exhibited by the nondependent user shifts to the compulsive use of increasingly higher doses when drug availability increases (Kramer et al. 1967, Gawin and Ellinwood 1989). Because this drug-intake escalation occurs in response to increased drug access in humans, drug addiction researchers study the behavioral aspects of psychostimulant dependence by using a well-established rodent self-administration model that induces drug-intake escalation through increased drug availability (Ahmed and Koob 1997, Kitamura et al. 2006, Wee et al. 2007, Rogers et al. 2008, Rocha and Kalivas 2010, Wee and Koob 2010). Experiments using this self-administration paradigm showed dramatic drug-intake escalation in rats provided with daily 6 h access to intravenous methamphetamine self-administration, whereas stable daily intake was observed in rats that were provided with daily 1 h access (Kitamura et al. 2006). In the same self-administration experiment, motivation for drug was tested by progressive-ratio (PR) self-administration, a paradigm in which each subsequent intravenous infusion of methamphetamine requires more and more lever presses. The results of the PR tests showed that while the rats with limited access to methamphetamine self-administration quickly lost motivation to continue seeking further drug-infusions, the rats provided with extended access to methamphetamine compulsively pressed the lever in order to receive more drug (Kitamura et al. 2006). These results suggest that only the extended-access group of rats developed dependence to methamphetamine. In a related experiment, treatment of dependent extended-access rats with κOR antagonist nor-BNI dramatically blocked drug-intake escalation and significantly decreased the motivational

response for methamphetamine, while κOR antagonism had no significant effect on the nondependent short-access group's daily intake of or motivation for methamphetamine (Whitfield et al. 2014). These results support the theory that κOR activation increases compulsive drug-seeking and -taking behavior in individuals with high availability to methamphetamine, thereby mediating their transition from occasional use to addiction (Wee and Koob 2010). While the results of Whitfield and colleagues showed that κOR antagonism prevented the initial emergence of the compulsive behaviors associated with extended access to methamphetamine, whether the same treatment will reverse these behaviors after the dynorphin/κOR system has already been upregulated remains to be elucidated.

Apart from the κOR itself, another eligible target for treating dynorphin-mediated methamphetamine dependence is transcription factor cyclic adenosine monophosphate response element-binding protein (CREB). CREB becomes an active transcription factor when it is phosphorylated by protein kinase A. This active form of CREB is known as phospho-CREB (p-CREB). It is theorized that mesolimbic p-CREB drives expression of dynorphin, leading to even further activation of κOR system (Unterwald et al. 2009). This theory is supported by a plethora of experiments that tie CREB activity to dysphoria and increased production of mesolimbic dynorphin. Viral vector-mediated overexpression of CREB in the NAc shell was shown to increase dynorphin mRNA expression in the NAc shell as well as induce well-established stress-response behaviors in rats (Carlezon et al. 1998, Pliakas et al. 2001, Muschamp et al. 2011). The assayed stress-response behaviors were blocked by both κOR antagonism with nor-BNI and viral vector-mediated overexpression of a dominant negative form of CREB (Carlezon et al.

1998, Pliakas et al. 2001, Muschamp et al. 2011). The results of research by Larson and colleagues additionally implicate CREB activity in compulsive cocaine self-administration as well. Viral vector-mediated overexpression of CREB in the NAc shell precipitated compulsive cocaine self-administration in rats, while small hairpin RNA-induced silencing of CREB in the NAc significantly decreased expression of dynorphin mRNA in the mesolimbic system of cocaine-dependent rats (Larson et al. 2011). Interpreted all together, these results suggest that increased κOR activity in the NAc phosphorylates and activates CREB. This increase in NAc CREB activity causes psychostimulant withdrawal-mediated dysphoria via upregulation of dynorphin in the mesolimbic system, which in turn decreases VTA dopamine release via negative feedback (Nestler 2004). Increased mesolimbic dynorphin also further activates κORs in the NAc, setting a perpetual spiral of dysphoria in motion. This downward spiral of withdrawal drives the compulsive drug-taking and -seeking behaviors exhibited by psychostimulant addicts. Although there is experimental evidence that links increased CREB activity in the NAc shell to dynorphin upregulation exhibited in cocaine addiction, evidence that implicates its involvement in chronic methamphetamine dependence is lacking (Carlezon et al. 2008, Larson et al. 2011).

My thesis-related research at the Koob lab explored two aspects of the involvement of the dynorphin/κOR system in the compulsive behavioral effects of chronic methamphetamine use. The first goal was to tie the compulsive drug-taking and -seeking behavior that is observed in animals with extended methamphetamine access to increased activity of CREB the NAc, which is an essential component in the dynorphin-mediated negative affect that characterizes psychostimulant withdrawal. The purpose of

Experiment 1 was to discover whether p-CREB immunoreactivity would be elevated in the NAc shell of animals with a history of extended access to methamphetamine self-administration or significantly escalated methamphetamine intake. Based on the results of several studies that implicate the elevated activity of CREB in the NAc shell with chronic use of cocaine, I hypothesized that escalated methamphetamine intake would coincide with increased CREB activity in the NAc shell region. The second goal was to reverse the compulsive methamphetamine-taking and -seeking behaviors that are theorized to be caused by upregulation of the dynorphin/κOR system in subjects with a history of extended access to the drug. The purpose of Experiment 2 was to discover whether systemic administration of κOR antagonist nor-BNI would block re-escalation and stress-induced reinstatement of methamphetamine self-administration. Based on support for the model that theorizes that methamphetamine withdrawal-induced dysphoria is driven by elevations in endogenous dynorphin, my colleagues and I hypothesized that κOR antagonism would reverse these previously conditioned behaviors. The overarching goal of these two experiments was to better understand the role of dynorphin/κOR system upregulation in the development of methamphetamine addiction.

Materials and Methods

Animals

32 male Wistar rats, (Charles River, Hollister, CA), each weighing between 250 and 300 g before surgery, served as subjects. Rats were housed in groups of two or three per cage in a temperature-controlled vivarium with a reversed 12:12 h light/dark cycle during which the lights were turned on at 8 pm. All experimental procedures were performed during the dark phase of the light/dark cycle. Food and water were available *ad libitum* throughout the experiments.

All animal use procedures were approved by The Scripps Research Institute Animal Care and Use Committee and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).

Surgery

Rats were anesthetized with 1-3% isoflurane mixed with oxygen; next they were implanted with silastic indwelling intravenous catheters (0.3 mm ID x 0.64 mm OD; Dow Corning Co., Midland, MI) into the right external jugular vein, as previously described (Wee et. al 2007). Catheters were flushed daily with a sterile saline solution containing heparin (30 USP units/mL) and antibiotic Timentin (30:1 ratio of ticarcillin disodium and clavulanate potassium, 100 mg/ml; GlaxoSmithKline, Philadelphia, PA).

Drugs

Methamphetamine hydrochloride (provided by The National Institute on Drug Abuse; Rockville, MD) was dissolved in 0.9% saline, and was self-administered intravenously at a dose of 0.05mg/kg/infusion. Nor-Binaltorphimine dihydrochloride (nor-BNI, provided by The National Institute on Drug Abuse) was dissolved in water and was injected subcutaneously at a dose of 30 mg/kg. Yohimbine (Sigma-Aldrich; St. Louis, MO) was dissolved in water and was injected intraperitoneally at doses of 1.0 and 2.5 mg/kg.

Methamphetamine Self-Administration

Methamphetamine self-administration sessions were held in operant chambers (28x26x20 cm; Med Associates Inc., St Albans, VT) that were placed in light- and sound-insulating cubicles. Experimental sessions were controlled and recorded by a PC computer. Each operant chamber had two retractable response levers attached to a sidewall and a stimulus light mounted above each lever. The two response levers emerged from the sidewall at the start of each self-administration session. Pressing the right response lever resulted in a syringe pump-mediated infusion of 0.1 ml of methamphetamine solution (0.05 mg/kg/infusion) administered over a period of 4 s. Each infusion was accompanied by illumination of the stimulus light above the active lever, which lasted throughout a 20 s time-out period. The shut off of the light at the end of the time-out period indicated availability of the next drug infusion. Responses on the active lever during the time-out period, as well as the inactive lever, were recorded but had no

other programmed consequences. When the session ended, the levers retracted into the sidewall.

All self-administration sessions were held on a daily basis on Monday through Friday every week. Catheter patency was tested by intravenous administration of short-acting barbiturate Brevital (methohexital sodium, 10 mg/ml, 2 ml/rat) whenever catheter failure was suspected during the self-administration.

Experiment 1: phospho-CREB immunohistochemistry

For experiment 1, a cohort of 12 rats was trained to self-administer 0.05 mg/kg/infusion of methamphetamine in 1 h sessions under a fixed-ratio (FR) 1 reinforcement schedule for 10 d. After acquisition of methamphetamine self-administration, the rats were split into two groups counterbalanced for the number of self-infusions during the last baseline session. One group was given 1 h access (short-access, or ShA) to drug self-administration per session, while the long-access (LgA) group was given 6 h access per session. For both groups, the escalation phase lasted for 13 sessions.

24 h after their final escalation session, the rats were anesthetized with 35% chloral hydrate (3 ml/450 g body weight, intraperitoneal administration) and perfused transcardially; first with cold 0.1 M phosphate buffered saline (PBS) and next with pH-neutral 4% paraformaldehyde solution. Brains were removed and cryoprotected in 30% sucrose solution, then sectioned coronally into 40 slices with a cryostat (Leica Microsystems, Buffalo Grove, IL). Sections were stored in PBS containing 1% sodium azide at 4°C before processing for immunohistochemistry (IHC). Brains from a group of 4 drug-naïve rats were processed in the same manner.

Immunoperoxidase staining of phospho-CREB (p-CREB) was measured in the nucleus accumbens (NAc) core and shell regions of the ventral striatum. Mouse monoclonal anti-phospho-CREB (Ser133) was used for labeling p-CREB immunoreactivity (Cell Signaling Technologies; Danvers, MA). All incubations were performed under agitation. Free-floating sections were rinsed with PBS, then incubated in 0.3% H2O2 for 45 min at room temperature to quench endogenous peroxidase activity. Nonspecific binding was blocked with a solution of 7.5% donkey serum, 2% bovine serum albumin and 0.3% Triton X-100 in PBS for 60 min at room temperature. Next, the sections were incubated with a 1:1000 dilution of p-CREB primary antibody (with 5% donkey serum and 0.5% TWEEN 20 in PBS) for 24 h at 4°C. Following incubation in the primary antibody, sections were rinsed again with PBS and incubated with horseradish peroxidase-conjugated secondary antibody (ImmPRESS Anti-Mouse Ig peroxidase polymer detection reagent, Vector Laboratories; Burlingame, CA) for 1 h. Immunoreactivity was visualized with 3,3' diaminobenzidine (DAB) staining using the Vector DAB Peroxidase Substrate Kit (Vector Laboratories; Burlingame, CA) for 3 min until specific peroxidase activity in the ventral striatum became visible. Omission of the primary antibody resulted in no specific staining, so it acted as a negative control for the immunoperoxidase staining. After rinsing again in PBS, the sections were mounted on glass slides, air-dried, dehydrated through a series of graded ethanol concentrations followed by clearing agent, and permounted.

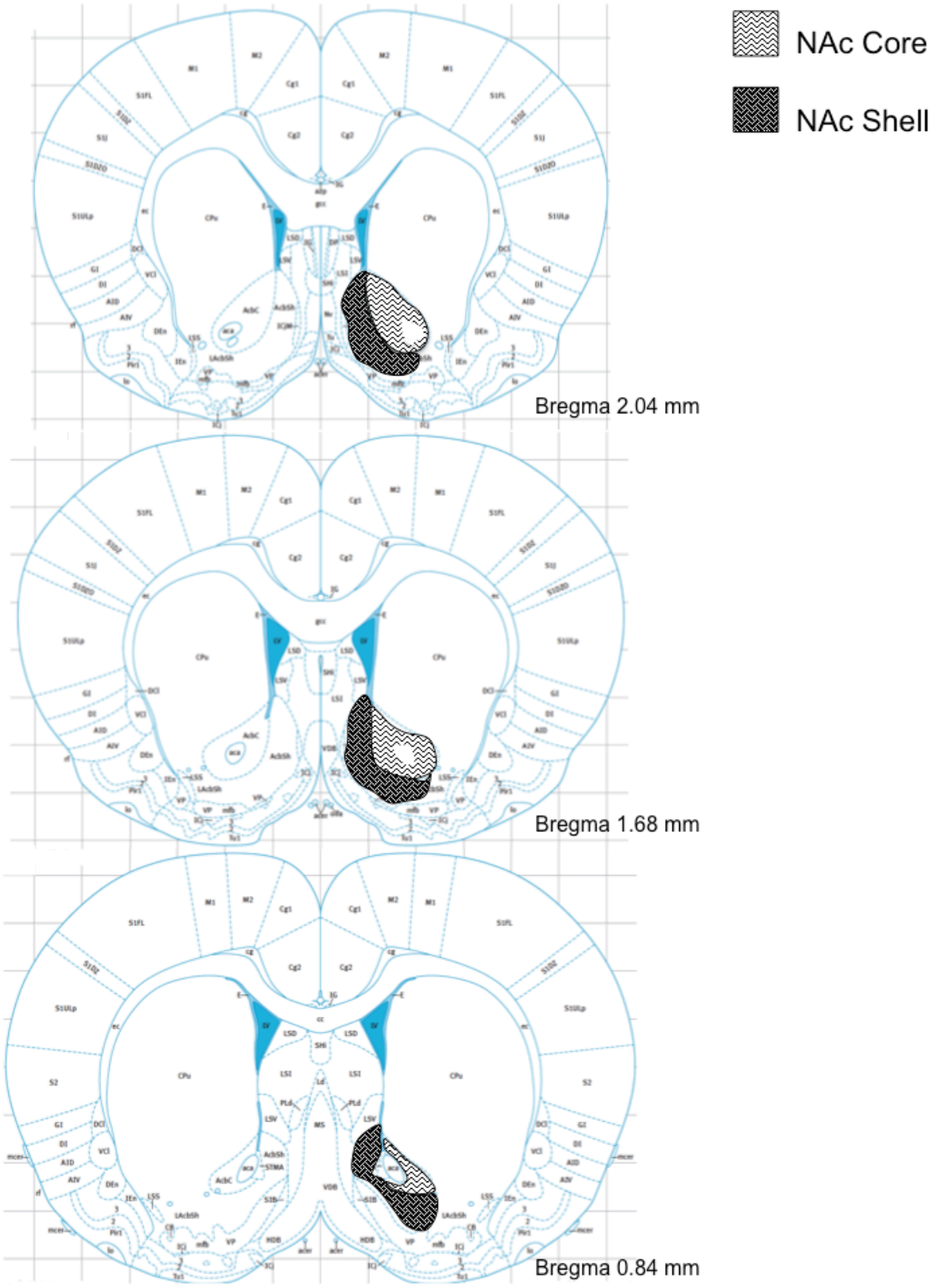


Figure 1. Schematic representation of the location of the NAc core and shell in coronal slices at 2.04 mm, 1.68 mm and 0.84 mm anterior to bregma. Images adapted from Paxinos and Watson (2009).

Group-blind analysis was performed to localize and quantify immunoreactivity (IR). DAB staining of the NAc was visualized with a Zeiss Axiophot photomicroscope and photographed with a Zeiss AxioCam-MRc digital camera. All photographs were taken under identical lighting and color intensity conditions. Specific staining in the NAc core and shell regions (AcbC and AcbSh: AP +2.04 to +0.84; Paxinos and Watson, 2009) from both hemispheres was quantified via measurement of integrated optical density. Integrated densities were obtained by converting each image to grayscale and inverting the image color using ImageJ software (National Institutes of Health), followed by standardization to background staining as observed in the anterior commissure. Measured integrated densities were averaged per rat, yielding one mean integrated density (MID) per rat, and then were expressed as normalized integrated density percentages compared to average integrated density measured among the drug-naïve rats.

The reported results exclude one rat that lost catheter patency and one rat that displayed nonspecific staining due to an issue that occurred during the perfusion.

Experiment 2: Re-escalation and reinstatement of methamphetamine self-administration

16 catheterized rats were trained to self-administer 0.05 mg/kg/infusion of methamphetamine in 1 h sessions under a fixed-ratio (FR) 1 reinforcement schedule for 10 d. Rats then entered the escalation phase of the experiment, during which they were allowed to self-administer 0.05 mg/kg/infusion of methamphetamine for 6 h per session under an FR 1 schedule. Following 21 sessions of self-administration escalation, all rats were subjected to a 45 d period of forced abstinence. At the end of the abstinence period, the rats were split into two groups counterbalanced for the number of self-infusions

during the last baseline session, and each rat received a systemic subcutaneous injection of either nor-BNI (30 mg/kg) or sterile water vehicle. 3 d following treatment, all rats entered the re-escalation phase (same reinforcement schedule, drug concentration and session length as initial escalation) of the experiment for 25 d to evaluate the effect of nor-BNI on re-escalation of drug intake. One rat died after the re-escalation phase.

Following the final re-escalation self-administration session, some of the re-escalated rats from each treatment group (n=9 total, 5 vehicle and 4 nor-BNI) entered the extinction phase of methamphetamine self-administration. In order to extinguish methamphetamine- seeking behavior, rats were placed in operant chambers but were not attached to an intravenous methamphetamine source. Active lever responses were recorded but had no programmed consequences. All rats met the criterion for extinction (fewer than 10 active lever responses per session) within 13 1 h extinction sessions.

Following extinction training, each rat underwent 2 yohimbine-stress-induced reinstatement tests. For the first test, the rats were injected intraperitoneally with yohimbine (2.5 mg/kg) 30 min prior to placement in operant chambers for a 1 h session, where active lever presses were recorded but had no consequences. After this reinstatement test, rats underwent an additional 2 extinction sessions prior to undergoing the second yohimbine-induced reinstatement test. The second reinstatement test used a lower dose of yohimbine (1 mg/kg) because the previous dose caused locomotor freezing.

Statistical analyses

All statistical analyses were performed using Prism 5.0 (GraphPad, San Diego, CA). The accepted level of significance for all tests was $p<0.05$ (two-tailed).

Experiment 1

The self-administration data were expressed as the mean number of infusions per total 6 h session (LgA rats) and the mean number of infusions in the first hour of the session (ShA and LgA rats). Methamphetamine self-administration was compared across the 13 escalation sessions using a two-way repeated-measures analysis of variance (ANOVA; access condition X session). When appropriate, post-hoc comparisons were performed using the Bonferroni multiple comparison posttest.

The difference in each NAc region's normalized MIDs across access condition groups was evaluated using one-way ANOVA with the Bonferroni multiple comparison post-hoc test. Correlations between behavioral data and MIDs for p-CREB immunoreactivity were evaluated by calculating Pearson product-moment correlation coefficients. For correlation analyses, error variance among the p-CREB MID data sets was homogenized by using a square root transformation.

Experiment 2

The self-administration data were expressed as the mean number of infusions per total 6 h session. Data for initial escalation of self-administration over time were evaluated using one-way repeated measures ANOVA. Re-escalation, extinction and reinstatement data were analyzed using two-way repeated-measures ANOVA (treatment X session). When appropriate, post-hoc comparisons were performed using either the paired Student's *t*-test or the Bonferroni multiple comparisons posttest.

Results

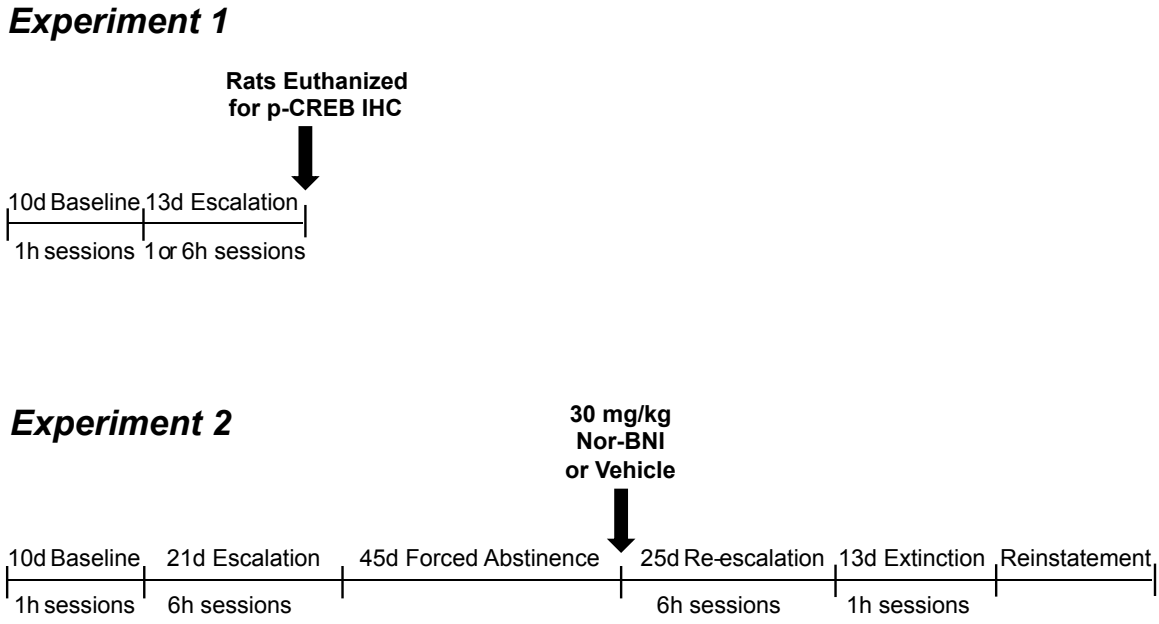


Figure 2. Schematic representation of experimental procedures used in experiments 1 and 2.

Experiment 1:

Figure 2 shows an overview schematic diagram of all experimental procedures, beginning with those used in Experiment 1. Methamphetamine self-administration increased significantly in LgA rats over the 13-session escalation phase of the experiment (Fig. 3). A two-way repeated measures ANOVA found a significant interaction between access condition (ShA or LgA) and session in methamphetamine intake within the 1st hour [$F_{(12,\ 96)}$=3.11, p<0.001; Fig. 3A] and in the total 6 h session [$F_{(12,\ 96)}$=9.80, p<0.0001, Fig. 3B]. Post-hoc tests determined that LgA rats significantly escalated their intake by the 11th session and maintained escalated intake until the final session for the 1st h of the sessions (Fig. 3A), and escalated their intake by the 9th session and maintained

escalated intake until the final session for the total 6 h session (Fig 3B). 24 h after the final self-administration session, all rats were euthanized and brain tissue was prepared for immunohistological detection of p-CREB. Fig. 4 shows representative sample photographs of p-CREB immunoreactivity (IR) in ventral striatum.

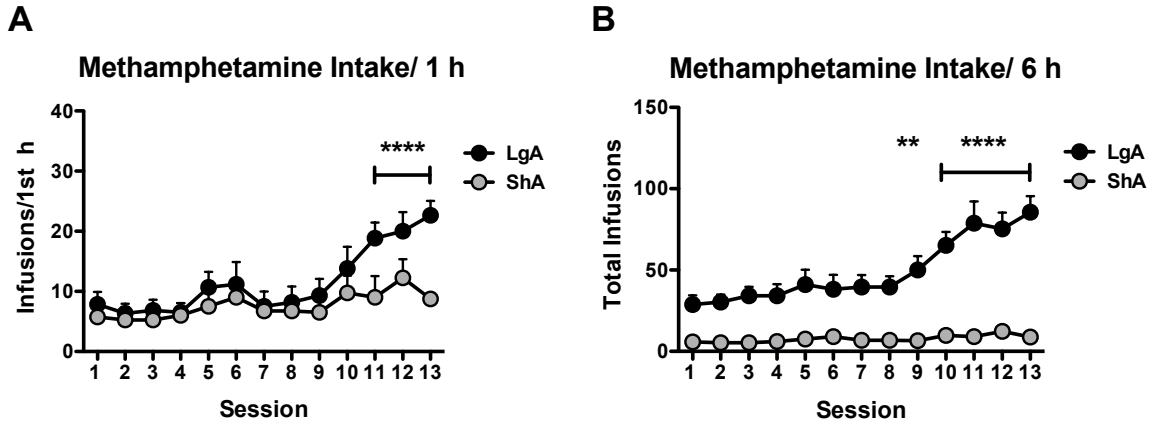


Figure 3. Effects of access condition on escalation of methamphetamine self-administration. **A)** Rats with a history of extended access to methamphetamine demonstrated escalation of drug intake during the 1st hour of self-administration. Post-hoc tests indicated that the LgA group escalated intake by the 11th session (****, $p<0.0001$) and continued to escalate intake until the final session. **B)** Rats with a history of extended access to methamphetamine demonstrated escalation of drug intake during the total 6 h sessions. Post-hoc tests indicated that the LgA group escalated intake by the 9th session (**, $p<0.01$) and continued to escalate intake until the final session (****, $p<0.0001$).

Normalized MID p-CREB immunoreactivity in both the NAc core and shell was modestly elevated for ShA rats and substantially elevated for LgA rats compared to drug-naïve rats (Fig. 5). Analysis of these access-based effects with one-way ANOVA demonstrated that these trends were not statistically significant for either the NAc core [$F_{(2,11)}=2.19$, $p>0.05$] or the NAc shell [$F_{(2,11)}=2.33$, $p>0.05$] .

Pearson product-moment correlation coefficients were computed to assess the relationship between the average number of infusions during the 1st hour of the last 3 sessions and the transformed p-CREB MIDs for the NAc core and shell. A trend of

positive correlation was found between escalated self-administered methamphetamine infusions and p-CREB IR in the NAc core [$r_{(8)}$=0.629, p=0.0515; Fig. 6A]. A significant positive correlation was found between escalated self-administered methamphetamine infusions and p-CREB IR in the NAc shell [$r_{(8)}$=0.639, p<0.05; Fig. 6B].

Pearson product-moment correlation coefficients were computed to assess the relationship between cumulative methamphetamine intake throughout all 13 escalation sessions and the transformed p-CREB MIDs for the NAc core and shell. There were significant positive correlations found between cumulative methamphetamine intake and p-CREB IR in both the NAc core [$r_{(8)}$=0.641, p<0.05; Fig. 7A] and shell [$r_{(8)}$=0.639, p<0.05; Fig. 7B].

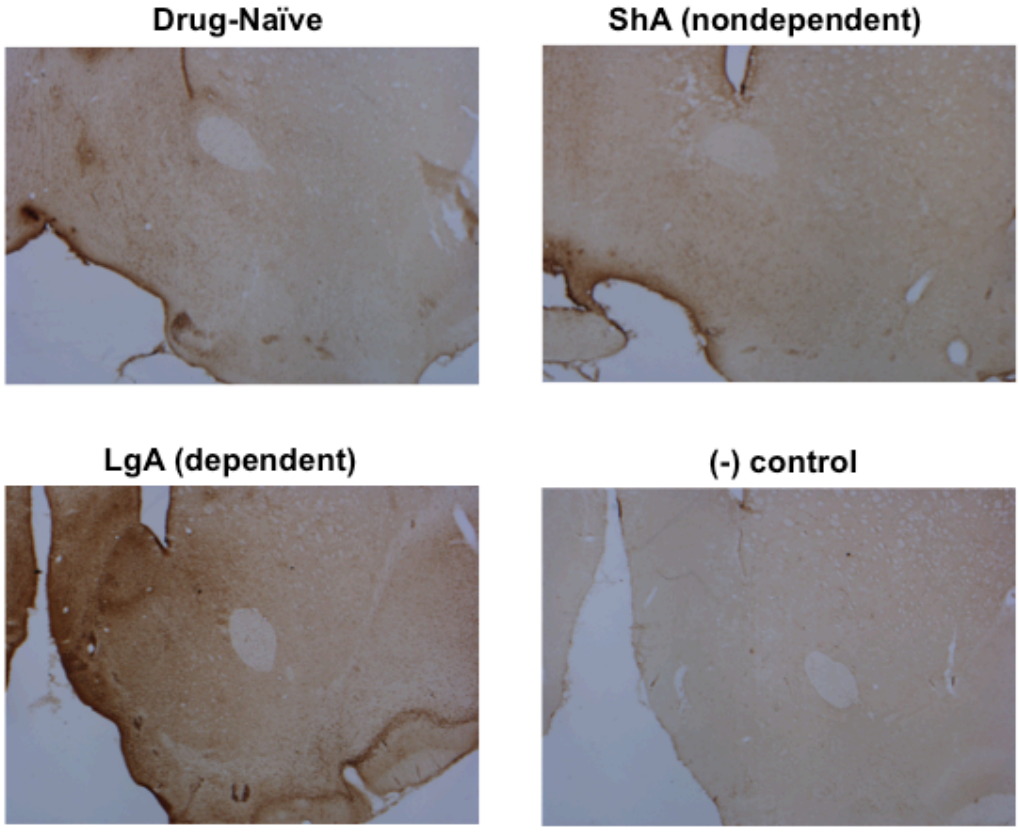
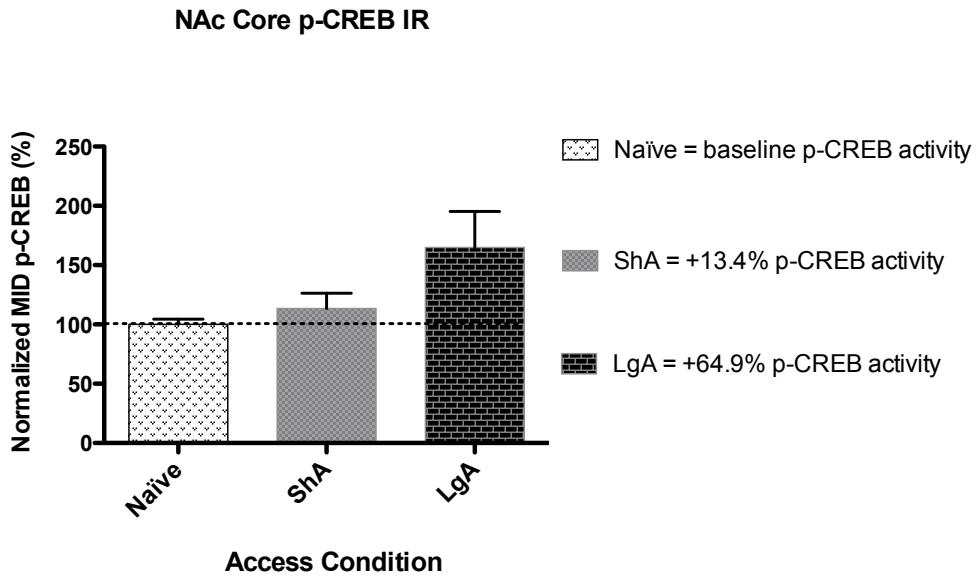


Figure 4. Representative photographs of p-CREB immunoreactivity in the ventral striatum.

A



B

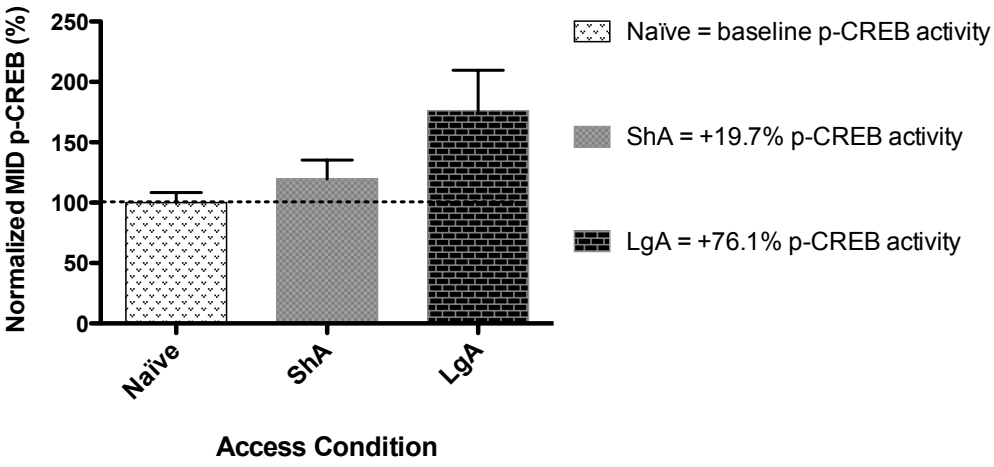


Figure 5. Effects of self-administration access condition on p-CREB immunoreactivity in the NAc. **A)** Rats with a history of extended access to methamphetamine showed a trend (p>0.05) of elevated p-CREB immunoreactivity in the NAc core. **B)** Rats with a history of extended access to methamphetamine showed a trend (p>0.05) of elevated p-CREB immunoreactivity in the NAc shell.

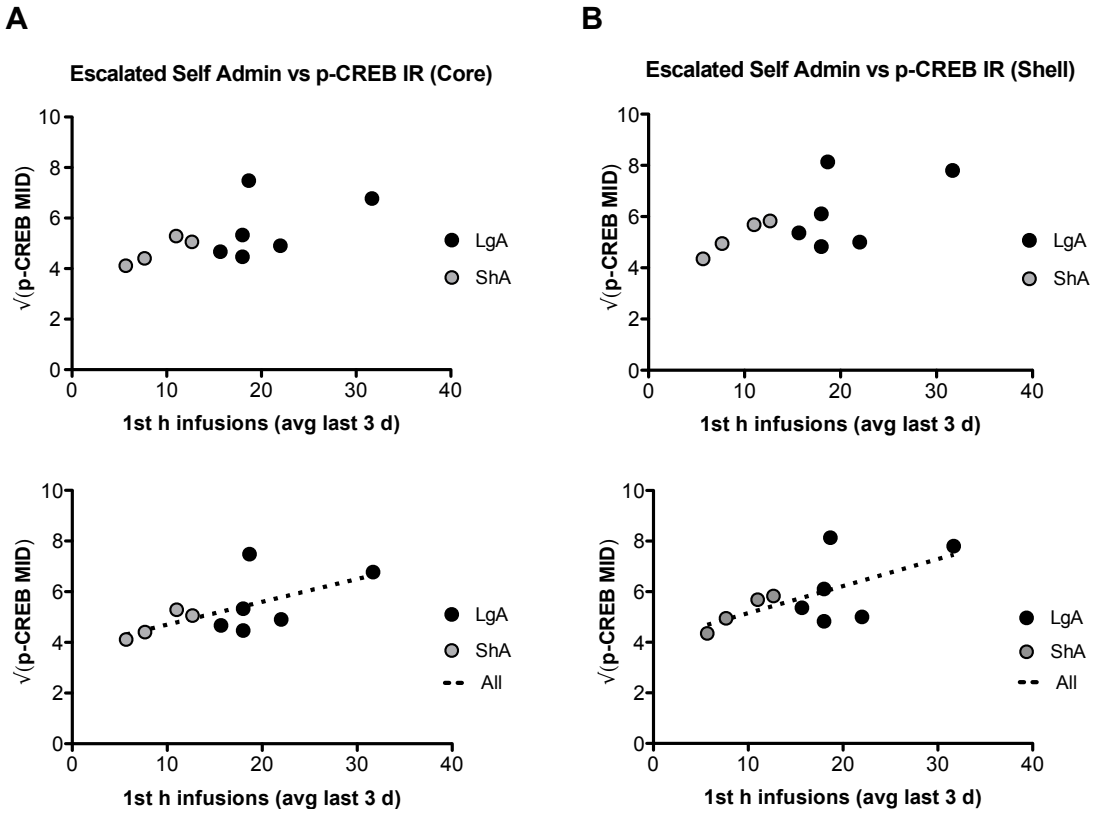


Figure 6. Correlation between escalated methamphetamine self-administration and p-CREB immunoreactivity in the NAc core **(A)** and shell **(B).** Positive correlations were found between infusions self-administered during the 1st h of the last 3 escalation sessions and p-CREB MIDs measured in the NAc core (p=0.0515) and NAc shell (p<0.05).

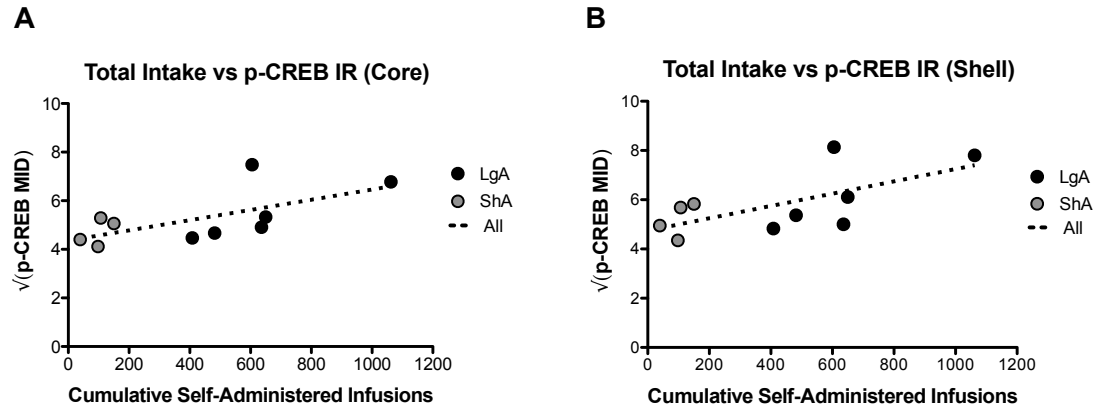


Figure 7. Correlation between cumulative methamphetamine intake during the escalation phase and p-CREB immunoreactivity in the NAc core **(A)** and shell **(B)**. Significant positive correlations were found between total methamphetamine intake and p-CREB MIDs measured in the NAc core (p<0.05) and NAc shell (p<0.05).

Experiment 2

The lower portion of Figure 2 shows a schematic diagram of the experimental procedures used in Experiment 2. Methamphetamine self-administration escalated by 65.5% over the 21 sessions of the initial escalation phase of the experiment (Fig. 8A). A one-way repeated measures ANOVA determined the overall escalation to be significant [$F_{(15, 20)} = 4.38$, $p<0.0001$]. Post-hoc tests determined that significant escalation of intake occurred during sessions 15 and 16 and re-emerged on session 21 (Fig. 8A). A paired t-test confirmed that significant escalation of intake occurred between the first and the last 3 sessions [$t_{(15)}=3.76$, $p<0.01$, Fig. 8B].

For the re-escalation phase data, a two-way repeated measures ANOVA comparing self-administration infusions over time found a significant main effect of nor-BNI treatment on methamphetamine intake during the 6 h session [$F_{(1,14)}=17.2$, $p<0.01$, Fig. 9]. Post-hoc tests determined that the vehicle-treated rats significantly re-escalated their intake by the 22nd session. In contrast, rats that had received pretreatment with nor-BNI did not experience re-escalation of self-administration over the 25 6 h sessions.

For the extinction phase, a two-way repeated measures ANOVA showed a significant interaction between treatment group and extinction session [$F_{(12, 84)}=2.43$, $p<0.01$], as well as a significant main effect of extinction session [$F_{(12, 84)}=9.57$, $p<0.0001$]. Post-hoc tests determined that there was a significant difference in methamphetamine-seeking active lever presses between the nor-BNI and vehicle treatment groups during the first day of extinction training ($p<0.001$, Fig. 10A).

To assess reinstatement of drug-seeking behavior, the number of yohimbine-stress-induced active lever presses was compared to average number of active lever

presses during the last 3 d of the extinction phase for each rat. A 2-way repeated-measures ANOVA found significant main effects of yohimbine test dose [$F_{(2,14)}$=17.09, p<0.001] and treatment group [$F_{(1,7)}$=10.04, p<0.05] on active lever presses, as well as a significant interaction between yohimbine dose and treatment group [$F_{(2,14)}$=6.85, p<0.01]. Post-hoc tests determined that while both doses of yohimbine significantly reinstated methamphetamine-seeking behavior in rats that had been pre-treated with vehicle, neither dose of yohimbine was able to induce reinstatement in rats that had received nor-BNI treatment over 40 days prior to the reinstatement testing (p<0.001, Fig. 10B).

Experiment 2, in part, is currently being prepared for submission for publication of the material. Whitfield Timothy W Jr., Grant Yanabel, Margolis Jessie, Koob George F. The thesis author was a researcher and co-author of this material.

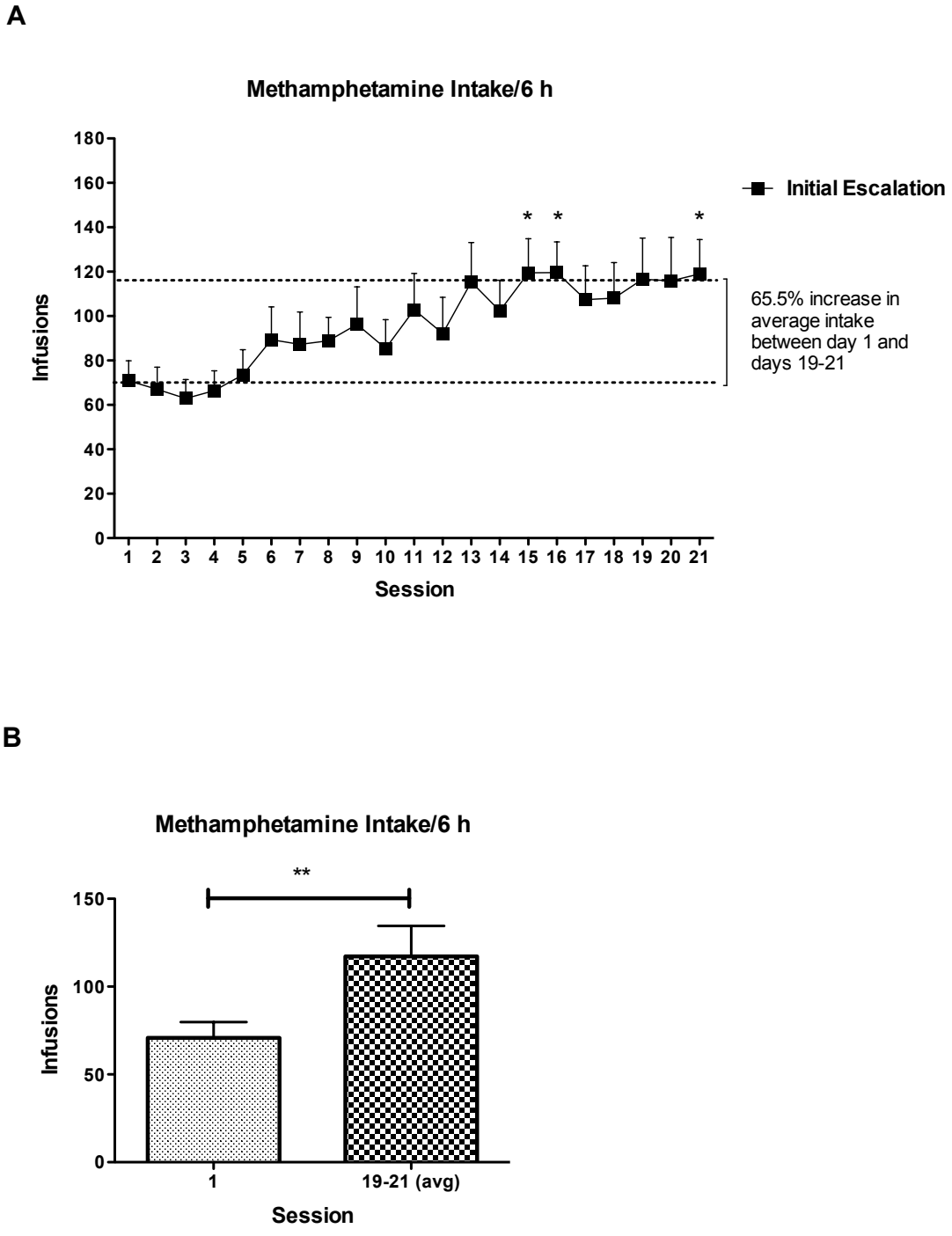


Figure 8. Escalation of methamphetamine self-administration **(A)** Significant escalation of methamphetamine self-administration occurred over the 21-session escalation phase ($p<0.001$). Post-hoc tests indicated that the cohort significantly escalated its collective intake on the 15th, 16th, and 21st sessions ($p<0.05$, *). **(B)** Post-hoc analysis showed significant escalation of self-administered intake between the 1st and last 3 sessions of the escalation phase ($p<0.01$, **).

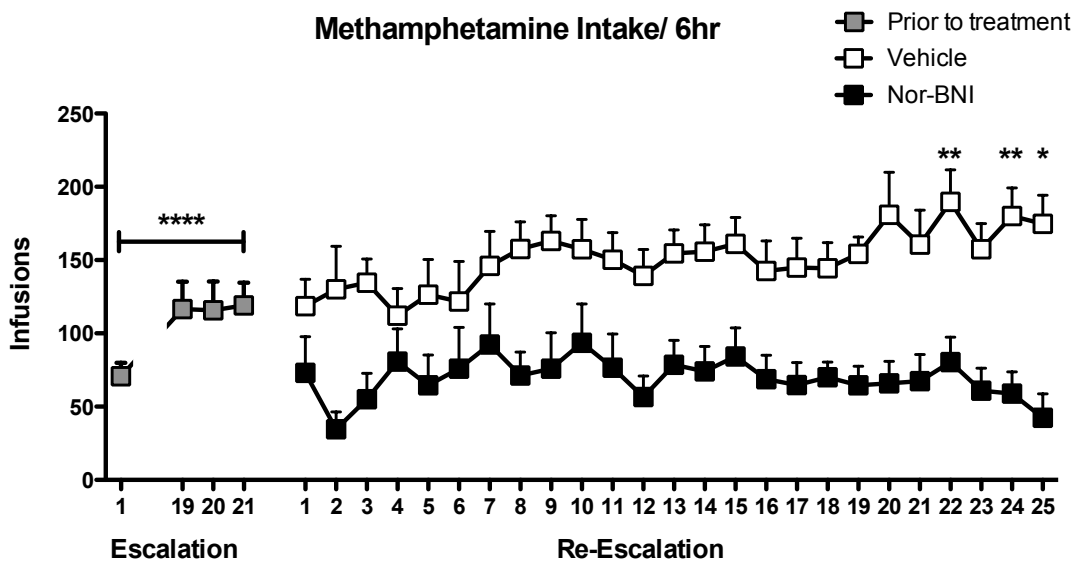


Figure 9. Re-escalation of methamphetamine self-administration. Following significant escalation that occurred during the 21-session initial escalation phase, methamphetamine self-administration increased significantly in only vehicle-treated rats over the 25 sessions of the re-escalation phase. Re-escalation was blocked in the nor-BNI treated rats.

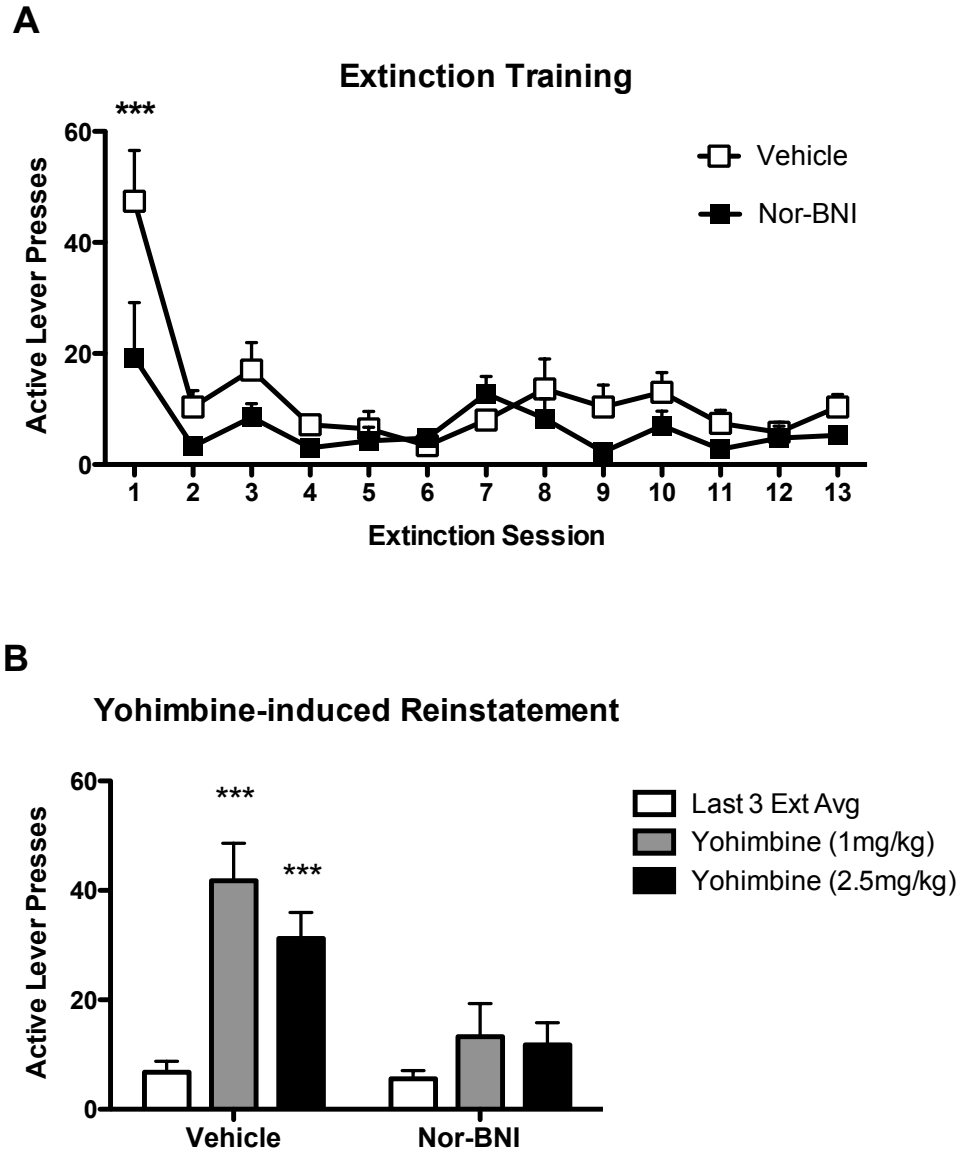


Figure 10. Extinction **(A)** and reinstatement **(B)** of methamphetamine seeking-behavior **(A)** The extinction of methamphetamine-seeking behavior over the course of 13 extinction sessions. Post-hoc tests showed a significant difference in active lever presses between treatment groups during session 1 of extinction ($p<.0.001$, ***). **(B)** The yohimbine-stress-induced reinstatement of methamphetamine-seeking behavior using two different doses of yohimbine. Post-hoc tests revealed that the vehicle-treated group experienced significant reinstatement at both doses, while reinstatement of the nor-BNI treated group was blocked at both doses ($p<.0.001$, ***).

Discussion

Phospho-CREB immunoreactivity in the NAc core and shell was upregulated proportionally to escalation of methamphetamine self-administration.

Psychostimulant-mediated D1 dopamine receptor activation in the NAc stimulates a cascade of signaling events that leads to phosphorylation-mediated transactivation of CREB, which causes alterations in gene expression (Konradi et al. 1994, Cole et al. 1995). In response to chronic exposure to amphetamine, p-CREB to binds to three CREs on the prodynorphin promoter, which subsequently activates expression of prodynorphin mRNA (Cole et al. 1995). Acute amphetamine and cocaine injections have been shown to increase p-CREB immunoreactivity in the striatum, particularly the NAc (Cole et al. 1995, Shaw-Lutchman et al. 2003, Walters et al. 2003). Chronic amphetamine injections yielded similar results (Cole et al. 1995, Shaw-Lutchman et al. 2003). These experiments did not differentiate between levels of p-CREB immunoreactivity in the core and shell regions of the NAc.

Whitfield and colleagues (2014) demonstrated that extended access to methamphetamine self-administration selectively increased prodynorphin immunoreactivity in the NAc shell. Immunohistological analysis of p-CREB immunoreactivity in the same animals failed to show a significant increase of CREB activity in either the core of shell region of the NAc. This finding could be due to a few reasons. The first explanation for why the experiment was unable to detect significant elevated CREB in the NAc could be because the number of subjects in each group was too low, as well as unequal between groups (4 drug-naïve, 4 short-access, and 6

extended-access animals). Variance within such small groups would make it difficult to attain a significant result. However, it is more likely that differences in NAc p-CREB immunoreactivity between limited-access and extended-access groups do not in fact parallel the region-specific elevations observed for prodynorphin. This could be because CREB phosphorylation has many roles in the brain apart from its involvement in upregulation of the dynorphin/κOR system. Another factor to consider is the relative position of CREB to prodynorphin in the cascade of events that is mediated by chronic D1 dopamine receptor activation in the NAc. Prodynorphin is downstream from CREB in the signal transduction cascade, so the effects of the cascade are amplified substantially more in prodynorphin than CREB. Therefore, it is possible that whenever a stimulus leads to the phosphorylation of only a few dozen molecules of CREB per cell in the NAc, the resulting elevation of dynorphin expression could be much more visually apparent and therefore be much easier to detect via immunoreactivity assay. Comparison of p-CREB versus total CREB expressed measured with a more sensitive and quantitative assay for expressed protein levels, such as immunoblot, might reveal a significant difference in CREB activity within the NAc regions.

While p-CREB immunoreactivity was not significantly upregulated in either region of the NAc in rats with a history of extended access to methamphetamine, a significant positive correlation was found between escalated self-administered methamphetamine (across all rats, regardless of access condition) and p-CREB immunoreactivity in the NAc shell, while a near-significant positive correlation was found for the NAc core. Since there were 6 or fewer rats per access condition group, it would be prudent to repeat the experiment with more subjects per group to confirm

whether this correlation between escalated methamphetamine self-administration and elevated CREB activity is indeed only significant within the NAc shell.

The results of this positive correlation do not provide evidence of causation; they merely tell us that self-administration escalates in tandem with the elevation of active CREB in the NAc. The correlation data do not tell us whether escalated methamphetamine self-administration indeed drove increased CREB activity in the NAc, as is posited by the chronic exposure model of dynorphin/κOR system upregulation, or if in fact it was inherently high levels of CREB activity in the NAc in certain animals that drove them to escalate their self-administration. Analysis of the relationship of self-administration data throughout the entire escalation phase and p-CREB immunoreactivity may provide more substantial evidence for a causal relationship in which chronic methamphetamine intake drives mesolimbic CREB activity. Comparison of cumulative self-administered methamphetamine intake to p-CREB immunoreactivity in the NAc core and shell revealed a significant positive correlation between these variables in both regions of the NAc. These findings suggest that increased cumulative methamphetamine intake may have been responsible for driving elevated CREB activity in the NAc. A different method of analysis for this relationship, such as use of linear regression rather than correlation coefficient or the use index of escalation during the 1st hour, rather than cumulative methamphetamine intake, may be more appropriate for answering this question of causality. Again, it would be prudent to repeat this experiment with more animals per group before making an interpretation based on the implicated role of elevated CREB in both regions of the NAc.

Expansion of the Experiment 1 protocol to include more treatment groups with $n>8$ per group could also provide more compelling evidence of the specific relationship between chronic methamphetamine self-administration and intra-NAc CREB. For instance, it could be valuable to observe the effects of systemically administered κOR antagonist nor-BNI and κOR agonist U50488, intra-NAc shell and intra-NAc core administration of nor-BNI, and intra-NAc shell and core administration of U50488 on the self-administration escalation data and the ratio of p-CREB to total CREB immunoreactivity in both NAc regions. Additionally, the causal link between changes in CREB activity and increased methamphetamine-taking and seeking behavior could be solidified with a study that manipulates CREB activity levels via viral vector-mediated overexpression of CREB and expression of dominant negative CREB in the NAc core and shell, followed by observation of the behavioral effects associated with extended access to methamphetamine self-administration.

The behavioral effects of extended access to methamphetamine were reversed by systemic κOR antagonism with nor-BNI.

Systemic treatment with nor-BNI significantly blocked re-escalation of methamphetamine self-administration in rats with a history of extended access to the drug. Since the rats had previously escalated their intake under extended access conditions, the nor-BNI-induced blockade of re-escalation showed that κOR antagonism attenuates the compulsive methamphetamine-taking behavioral effect of extended access to methamphetamine. Furthermore, following extinction of methamphetamine-taking behavior, the same group that had been previously treated with nor-BNI experienced

significant blockade of yohimbine-stress-induced reinstatement, while drug-seeking behavior was reinstated in the rats that received vehicle. The nor-BNI-induced blockade of self-administration reinstatement showed that κOR antagonism is able to significantly reduce stress-induced re-emergence of previously conditioned compulsive methamphetamine-seeking behavior.

Interpreted together, these results suggest that the dynorphin/κOR system is a potential target for reversing the compulsive behavioral aspects of methamphetamine-taking and -seeking in subjects with a history of chronic methamphetamine abuse. This discovery adds to Whitfield and colleagues' finding that both systemic administration of nor-BNI and intracranial injection of nor-BNI into the NAc shell prevented the emergence of the escalation of methamphetamine self-administration (Whtifield et al. 2014). Collectively, these results suggest that the dynorphin/κOR system could potentially serve as a therapeutic target that not only prevents initial emergence of compulsive behavioral effects associated with chronic methamphetamine use, but also decreases the likelihood of relapse precipitated either by temptation due to increased drug availability or by stress.

Although κOR antagonism with nor-BNI blocked the re-emergence of compulsive drug-taking and seeking behavior in rats, it is not likely to be a good candidate for treatment of methamphetamine addiction in humans. JDTic, a long-lasting κOR-selective antagonist that is functionally very similar to nor-BNI, was advanced to phase 1 clinical trials for cocaine abuse, but the study was terminated due to the adverse effect of ventricular tachycardia (Urbano et al. 2014). This occurrence suggests that use of a long-acting κOR antagonist to treat methamphetamine addiction is too dangerous to be a

viable treatment option for humans. Therefore, it may be worthwhile to explore other components involved in the upregulation of the dynorphin/κOR system as potential therapeutic targets for reversing the behavioral effects associated with chronic methamphetamine addiction in humans. As suggested by the findings of Experiment 1, CREB within the NAc may prove to be a worthwhile candidate for such a therapeutic target; however, before this option can be considered, there will need to be more compelling evidence that links this component of dynorphin/κOR system dysregulation to methamphetamine addiction, and the functional differences of inhibition of CREB activity in the NAc core versus shell regions will have to be successfully elucidated. In conclusion, the combined results of this thesis preliminarily identify NAc CREB as a potential therapeutic target for reversal and prevention of relapse of the compulsive behavioral effects associated with chronic exposure-induced methamphetamine addiction.

REFERENCES

Ahmed S and Koob G. (1998) Transition from moderate to excessive drug intake: change in hedonic set point. *Science* 282, 298–300

Bechara A and van der Kooy D. (1987) Kappa receptors mediate the peripheral aversive effects of opiates. *Pharmacol Biochem Behav* 28, 227-233.

Bruchas M, Land B, Chavkin C. (2009) The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Research* 1314, 44-55.

Bruchas M and Chavkin C. (2010) Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacol* 210*,* 137-147.

Carlezon W Jr, Thome J, Olson V, Lane-Ladd S, Brodkin D, Hiroi N, Duman R, Neve R, Nestler E. (1998) Regulation of cocaine reward by CREB. *Science* 282, 2272-2275.

Chavkin C, James I, Goldstein A. (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* 21*,* 413-5.

Cole R, Konradi C, Douglass J, Hyman S. (1995) Neuronal adaptation to amphetamine and dopamine: Molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* 14*,* 813-819.

Di Chiara G and Imperato A. (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *PNAS* 85, 5274-5278.

Diagnostic and Statistical Manual of Mental Disorders (4th ed., text rev.) doi: 10.1176/appi.boks. 97815623754.394906

Fischer J and Cho A. (1979) Chemical release of dopamine from striatal homogenates: Evidence for an exchange diffusion model. *J Pharmacol Exp Ther* 208, 203-209.

Gawin F and Ellinwood E. (1989) Cocaine dependence. *Annual Rev Med* 40, 149-161.

Harwood H, Fountain D, Livermore G. (1998) The Economic Costs of Alcohol and Drug Abuse in the United States. Report prepared for the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, U.S. Department of Health and Human Services. NIH

Heikkila R, Orlansky H, Cohen G. (1974) Studies on the distinction between uptake inhibition and release of [3H]dopamine in rat brain tissue slices. *Biochem Pharmacol* 24, 847-852.

Hodos W. (1961) Progressive ratio as measure of reward strength. *Science* 134, 943-944.

Ito R, Robbins T, Everitt B. (2004) Differential control over cocaine-seeking behavior by nucleus core and shell. *Nat Neurosci* 7, 389-397.

Jones S, O'Dell S, Marshall J, Wightman R. (1996) Functional and anatomical evidence for different dopamine dynamics in the core and shell of the nucleus accumbens in slices of rat brain. *Synapse* 23*,* 224-231.

Kitamura O, Wee S, Specio S, Koob G, Pulvirenti L (2006) Escalation of methamphetamine self-administration in rats: A dose-effect function. *Psychopharmacol* 186*,* 48-53.

Konradi C, Cole R, Heckers S, Hyman S. (1994) Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *J Neuro* 14, 5623-5634.

Koob G and Le Moal M. (2006) *Neurobiology of Addiction*. (London: Elsevier/Academic Press), 1-51, 69-108.

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

Kosten T and Newton T. (2012) Epidemiology and psychiatric comorbidity, in *Cocaine and Methamphetamine Dependence: Advances in Treatment.* (Washington, DC: American Psychiatric Association), 1-7.

Kramer J, Fischman V, Littlefield D. (1967) Amphetamine abuse: Patterns and effects of high doses taken intravenously. *JAMA* 201, 305-309.

Land B, Bruchas M, Lemos J, Xu M, Melief E, Chavkin C. (2008) The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J Neurosci* 28*,* 407-14.

Larson E, Graham D, Arzaga R, Buzin N, Webb J, Green T, Bass C, Neve R, Terwilliger E, Nestler E, Self D. (2011) Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. *J Neurosci* 31*,* 16447-16457.

McLaughlin J, Marton-Popovici M, Chavkin C. (2003) κ-opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci* 23*,* 5674-5383.

Meredith G. (1999) The synaptic framework for chemical signaling in the nucleus accumbens. *Ann N Y Acad Sci* 877, 140-156.

Muschamp J, Van't Veer A, Parsegian A, Gallo M, Chen M, Neve R, Meloni E, Carlezon W Jr. (2011) Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. *J Neurosci* 31, 3095-3103.

Nabeshima T, Katoh A, Wada M, Kameyama T. (1992) Stress-induced changes in brain Met-enkephalin, Leu-enkephalin and dynorphin concentrations. *Life Sci* 51*,* 211–217.

Nestler E. (2004) Molecular mechanisms of drug addiction. Neuropharmacol *47*, 24-32.

Newton S, Thome J, Wallace T, Shirayama Y, Schlesinger L, Sakai N, Chen J, Neve R, Nestler E, Duman R. (2002) Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J Neurosci* 24*,* 10883-10890.

Paxinos G, Watson C. (2009) *The rat brain in stereotaxic coordinates,* compact 6th ed (London: Academic Press).

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

Rocha A and Kalivas P. (2010) Role of the prefrontal cortex and nucleus accumbens in reinstating methamphetamine seeking. *Eur J Neurosci* 31, 905-908.

Rogers J, de Santis S, See R. (2008) Extended methamphetamine self-administration enhances reinstatement of drug seeking and impairs novel object recognition in rats. *Psychopharmacol* 199, 616-619.

Shaw-Lutchman T, Impey S, Storm D, Nestler E. (2003) Regulation of CRE-mediated transcription in mouse brain by amphetamine. *Synapse* 48, 10-17.

Shippenberg T, Rea W. (1997) Sensitization to the behavioral effects of cocaine: modulation by dynorphin and kappa-opioid receptor agonists. *Pharmacol Biochem Behav* 57, 449-455.

Simpson J, Wang J, McGinty J. (1995) Repeated amphetamine administration includes a prolonged augmentation of phosphorylated cyclase response element-binding protein and Fos-related antigen immunoreactivity in rat striatum. *Neurosci* 69*,* 441-457.

Smiley P, Johnson M, Bush L, Gibb J, Hanson G. (1990) Effects of cocaine on extrapyramidal and limbic dynorphin systems. *J Pharmacol Exp Ther* 253, 938-943.

Snyder S and Coyle J. (1969) Regional differences in H3-dopamine uptake into rat brain homogenates. *J Pharmacol Exp Ther* 165, 78-86.

Svingos A, Colago E, Pickel V. (1999) Cellular sites for dynorphin activation of kappa-opioid receptors in the rat nucleus accumbens shell. *J Neurosci* 19, 1804-1813.

Unterwald E, Howells R. (2009) Upregulation of opioid receptors, in *Opiate Receptors and Antagonists: From Bench to Clinic*. (New York: Humana Press), 274.

Urbano M, Guerrero M, Rosen H, Roberts E. (2014) Antagonists of the kappa opioid receptor. *Bioorganic Med Chem Lett*, doi: 10.1016/j.bmcl.2014.03.040.

Volkow N, Fowler J, Wang G. (2002) Role of dopamine in drug reinforcement and addiction in humans: results from imaging studies. *Behav Pharmacol* 13, 355-366.

Voorn P, Gerfen C, Groenewegen H. (1989) Compartmental organization of the ventral striatum of the rat: Immunohistochemical distribution of enkephalin, substance P, dopamine and calcium-binding protein. *Comp Neurol* 289, 189–201.

Walters C, Kuo Y, Blendy J. (2003) Differential distribution of CREB in the mesolimbic dopamine reward pathway. *J Neurochem* 87, 1237-1244.

Wee S, Specio S, Koob G. (2007) Effects of dose and session duration on cocaine self-administration in rats. *J Pharmacol Exp Ther* 320*,* 1134-1143.

Wee S, Orio L, Ghirmai S, Cashman J, Koob G. (2009): Inhibition of kappa opioid receptors attenuated increased cocaine intake in rats with extended access to cocaine. *Psychopharmacol* 205, 565-575.

Wee S and Koob G. (2010): The role of the dynorphin–κ opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacol* 210, 121-135.

Whitfield T, Wee S, Schlosburg J, Vendruscolo L, Edwards S, Gould A, Grant Y, Crawford E, Koob G. (2014): Kappa opioid receptor antagonism with Nor-binaltorphimine (NorBNI) prevents the emergence of compulsive behaviors associated with extended-access methamphetamine self-administration, submitted to *J Neurosci.*

Wise R and Bozarth M. (1984) Brain reward circuitry: Four critical elements "wired" in apparent series. *Brain Res Bull* 2*,* 203-208.